PHOSPHORYLATION REACTION OF CATION TRANSPORT ATPases.

A STUDY ON PURIFIED AND RECONSTITUTED ENZYMES.

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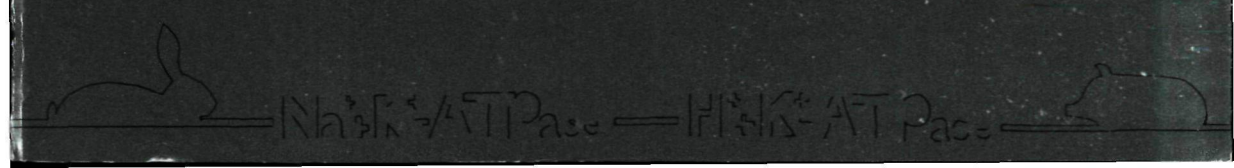

PHOSPHORYLATION REACTION OF CATION TRANSPORT ATPases.
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een wetenschappelijke proeve op het gebied van de
NATUURWETENSCHAPPEN,
in het bijzonder de Biochemie

proefschrift
ter verkrijging van de graad van doctor
aan de Katholieke Universiteit te Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
vrijdag 27 april 1990
des namiddags te 1.30 uur precies

door

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geboren op 21 november 1957
te Schijndel

1990
Gedrukt bij Offsetdrukkerij Kanters bv, Alblasserdam

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Aan Carin

Voor mijn ouders

The investigations described in this thesis were carried out at the Department of Biochemistry, University of Nijmegen, The Netherlands, under the direction of Prof. Dr. J.J H H.M. De Pont and at the Max-Planck Institut für Biophysik in Frankfurt, F.R.G. under the direction of Prof. Dr E Bamberg Financial support was obtained from the Netherlands Organisation for Scientific Research (N W O), through the Netherlands Biophysics Foundation (Stichting voor Biofysica).

CONTENTS

Dankwoord

Via deze weg wil ik iedereen, die op enigerlei wijze heeft bijgedragen aan het tot stand komen van dit proefschrift hartelijk bedanken. In het bijzonder betreft dit Marlea van Drunen, Candida van Hooydonk, Jan van Kleef, Albert Kamps en Henk Koster, voor de bijdrage die zij geleverd hebben in het kader van hun bij- of hoofdvak of HBO-stage.

Natuurlijk dank ik iedereen van de Afdeling Biochemie voor de prettige werksfeer. Vooral waren de gezellige uurtjes binnen en buiten het lab. met Arno Skrabanja, de nuttige suggesties en de hulp bij de lay-out van dit proefschrift door Herman Swarts en de discussies met Tom van Uem mij erg waardevol.

Weiter möchte ich Prof. Dr. Ernst Bamberg und seiner Arbeitsgruppe vom Max-Planck Institut für Biophysik in Frankfurt herzlich danken für die mir gebotene Möglichkeit sämtliche Experimente in ihrem Labor durch zu führen. Im Besonderen danke ich dabei Afra Hüby für die Einführung in die Bilayer Experimente und die herlichen Frühstücke, Klaus Fendler und Georg Nagel für viele Discussionen in und ausserhalb des Labors und Hans-Jürgen Butt für die gute Zusamenarbeit. Die ganze Gruppe hat durch ihre besondere Gastfreundschaft die vielen Aufenthalte in Frankfurt zu einem sehr angenehmen Erlebnis gemacht.

Auch Dr. Ernst Grell und Sabine Schmitt vom Max Planck Institut für Biophysik möchte ich danken für die längere Zusammenarbeit im Bezug auf die *lin*-benzo Experimente.

Mijn bijzondere dank gaat uit naar mijn ouders, zonder wie die alles niet mogelijk zou zijn geweest en die nu eindelijk eens kunnen zien waar al die jaren toe geleid hebben.

En last but absolutely not least, bedankt Carin voor de vele vele uren geduld en steun gedurende de afgelopen vier jaar.

Chapter 1

Introduction

Plasma membrane and ion transport.

In all animal cells the ionic composition of the cytosolic and extracellular compartments is strongly different. In the extracellular fluid high concentrations of Na^+ and low concentrations of K^+ are present, whereas the opposite ratio is found for the two cations in the cytosolic compartment. Although the plasma membrane, containing a lipid bilayer as basic structure, forms a firm barrier for charged molecules there is a considerable permeability for these species provided by the presence of specific channels and carriers.

Some intrinsic proteins which span the plasma membrane allow hydrophilic compounds to cross the membrane. The direction of the transport of a distinct compound is generally down the electrochemical gradient and is therefore defined as a passive process. The specificity of the channel for different hydrophilic compounds provides selective permeability. The permeability (leakage) of the membrane can be regulated by means of a triggered opening and closing of the channels. An alternative for passive transport across the membrane is given by carrier proteins. Carriers are able to facilitate diffusion of hydrophilic substances across the membrane by binding the compound and shielding the polar or charged groups of it. The permeability of the cell membrane containing carriers and channels is large enough to dissipate the gradients within short time. The selective permeability of the cell membrane for various ions together with the ion gradients is responsible for the membrane potential which can be described by the Goldman equation (Goldman, 1943). To maintain the gradients active pumping is necessary. The ion-transport is directed against the electrochemical gradients and must therefore be active.

An enzyme system, spanning the membrane takes care of the active transport of Na^+ and K^+ across the plasma membrane. This enzyme splits the γ-phosphate of ATP and uses the released energy to drive the cation transport. In this process chemical energy is converted into cation motion (Jencks 1983). This ATP hydrolysing, Na^+ and K^+ pumping, enzyme is called $(Na^+{+}K^+)$-ATPase, (sodium, potassium and magnesium stimulated adenosine triphosphatase (EC 3.6.1.37)) or Na^+-pump.

Two related membrane-bound ATPases have been isolated from animal cells: the so called $(H^+{+}K^+)$-ATPase (proton, potassium and magnesium stimulated adenosine triphosphatase (EC 3.6.1.36) and calcium and magnesium stimulated adenosine triphosphatase, or Ca^{2+}-ATPase (De Meis and Vianna 1979).

The latter two ATPases have much in common in structure and function with $(Na^+{+}K^+)$-ATPase, but differ with respect to the cations they can transport. $(H^+{+}K^+)$-ATPase is responsible for acidification in the stomach by exchanging H^+ against K^+. It is located in the secretory vesicles of the gastric parietal cell, whereas $(Na^+{+}K^+)$-ATPase is present in all animal cells. $(H^+{+}K^+)$-ATPase is capable of maintaining a proton gradient of 10^6 across the secretory membrane.

Two different types of Ca^{2+}-ATPase have been characterized: one which is present in the plasma membrane, responsible for the maintenance of low cytosolic Ca^{2+} concentrations and one occurring in membranes of organelles responsible for the storage of Ca^{2+}, like the sarcoplasmic and endoplasmic reticulum and the recently postulated calciosomes (Volpe et al 1988).

Historical perspective of $(Na^+{+}K^+)$-ATPase and $(H^+{+}K^+)$-ATPase

$(Na^+{+}K^+)$-ATPase

The idea of a sodium pump present in the mammalian cell has already been introduced in 1941 (Dean 1941). In this period it was observed that a suspension of red blood cells looses K^+ and takes up Na^+ upon cooling to 0°C, whereas it takes up K^+ and extrudes Na^+ against their chem-

ical gradients upon subsequent incubation at 37°C. This observation led to the idea of active transport. A few years later it was shown that Na^+ and K^+ transport were coupled to each other (Steinbach 1951, Hodgkin and Keynes 1955, Glynn 1956). Thereafter it was reported that ATP supports Na^+ and K^+ transport in erythrocyte ghosts (Gardos 1954, Caldwell and Keynes 1957).

In 1957 the discovery of an enzyme from the membrane fraction of the Carcinus maenas nerve was reported (Skou 1957). This protein was able to hydrolyse ATP, provided that Na^+, K^+ and Mg^{2+} were present. This suggested that this ATPase was responsible for active ion transport. This was supported by the findings of Skou (1960) showing that cardiac glycosides, known inhibitors of active Na^+ transport in erythrocytes (Schatzmann 1953), inhibited this enzyme. With the method of Jørgensen (1974a) it became possible to obtain the enzyme ($(Na^+{+}K^+)$-ATPase) in a highly purified form with high specific activity. The purified enzyme containes a certain amount of phospholipids originating from the plasma membrane. With these membrane fragments containing $(Na^+{+}K^+)$-ATPase as the major protein component many structural and functional studies have been carried out.

Since the enzyme in the broken membrane sheets had lost its compartimentalisation, transport studies were not possible with this preparation. Transport studies, however, have been carried out with intact erythrocytes (Garrahan and Glynn 1967 a,b,c,d,e), erythrocyte ghosts (Glynn and Karlish 1976) and native membrane vesicles (Forbush 1982a, 1984). The disadvantage of the above preparations is the presence of other membrane-bound proteins which may interfere in the transport measurements and obscure interpretations. The need for $(Na^+{+}K^+)$-ATPase containing vesicles free of contaminating proteins led to the development of reconstituted $(Na^+{+}K^+)$-ATPase. In this system highly purified $(Na^+{+}K^+)$-ATPase is incorporated in artificial lipid vesicles. Transport experiments with these proteoliposomes have been carried out.

$(H^+{+}K^+)$-ATPase

It has been known for more than a century that the acidification of the stomach is due to the secretion of hydrochloric acid (Prout 1824). The pump and the energy source of the pump however remained obscure until 1974, when Durbin et al (1974) proved that ATP could act as a direct energy source for acid secretion in the parietal cell. Another important step in the direction of discovery of the proton pump was the observation that the presence of K^+ was a prerequisite for H^+ secretion by the parietal cell (Harris et al 1958, Sedar and Wiebelhaus 1972). The isolation of a K^+ dependent and ouabain insensitive p-nitrophenylphosphatase (pNPPase) activity (Forte et al 1967) preceded the discovery of a gastric K^+-stimulated ATPase activity in gastric membrane vesicles (Ganser and Forte 1973) to which the pNPPase activity was purified in parallel. The link between the K^+- stimulated ATP-ase activity and proton transport was made by the observation that addition of ATP to closed gastric membrane vesicles induced an alkalinization of the medium, indicating proton uptake by the vesicles (Lee et al 1974). Finally Sachs et al (1976, 1977) completed the view by demonstrating that the H^+ accumulation in the vesicles was the result of ATP driven exchange of intravesicular K^+ for H^+. Although a direct proof that H^+ activates this enzyme was lacking, the enzyme was coined $(H^+{+}K^+)$-ATPase, given the analogy with $(Na^+{+}K^+)$-ATPase.

Physiological functions of $(Na^+{+}K^+)$-ATPase and $(H^+{+}K^+)$-ATPase

The main function of $(Na^+{+}K^+)$-ATPase is the active transport of K^+ into and Na^+ out of the cell, leading to maintainance of ion gradients (mainly $\Delta\mu_{Na^+}$) and the membrane potential as mentioned above. This might have different implications on the physiology of the cell depending on its type. In single cells e.g. erythrocytes and leucocytes the cation pump has an important function in the regulation of the osmotic value and cytosolic K^+ concentration. Another (indi-

rect) role is played by the $(Na^+ + K^+)$-ATPase in the intestinal tract in and the kidney providing the gradient for the Na^+ driven uptake of nutrients such as glucose and amino acids, metabolites such as citrate or succinate, ions like H^+, Ca^{2+}, phosphate or chloride and solutes by the cell. This kind of transport driven by a Na^+ gradient is known as uphill or secondary active transport. In excitatory systems the Na^+-pump plays a role in the restoration of the membrane potential after depolarisation and setting up the excitability of the cell. In secretory systems the Na^+ pump is involved in the excretion and absorption of salts, water and solutes. In cardiac and smooth muscle cells the presence of the Na^+/Ca^{2+} exchanger allows the Na^+-pump to indirectly control the cytosolic Ca^{2+} concentration. In salt glands of marine birds and the rectal glands of elasmobrach fish the major function of $(Na^+ + K^+)$-ATPase is to remove part of the salt of the sea water consumed by these animals.

The function of $(H^+ + K^+)$-ATPase is secretion of H^+ from the parietal cells into the lumen of the stomach. Here the hydrochloric acid has a double function. An acid medium is required for conversion of pepsinogen to pepsin, a proteolytic enzyme responsible for the primary digestion steps of proteins. The second function, however, the bacteriostatic effect of the acid medium is considered to be at least as important as the first one. The acidity of the stomach prevents bacteria from entering the intestine. Several years ago a $(H^+ + K^+)$-ATPase-type enzyme has been isolated from mammalian colon (Gustin and Goodman 1981). The function of acidification in this organ is unknown up till now.

Regulation

The regulation of $(Na^+ + K^+)$-ATPase must be finely controled (see for overviews: Jørgensen 1986, Rossier et al 1987 and Presley 1988). Therefore fast and long term mechanisms exist for the regulation. Since $(Na^+ + K^+)$-ATPase occurs in every animal cell a local control must exist in parallel with the control for the whole organism. Local regulation is under control of ligands: monovalent cations, ATP and analogues or cardiac glycosides and their putative physiological counterparts (endo-ouabain (Haupert et al 1985)). The main monovalent cation controling the $(Na^+ + K^+)$-ATPase activity is Na^+. Since the intracellular Na^+ concentration is too low to saturate the Na^+-pump, an increase in cytosolic Na^+ concentration will speed up the pump rate. The effect of Na^+ is fast (in the second range). K^+ plays a minor role in regulation of the pump. Endogenous regulators of $(Na^+ + K^+)$-ATPase which function in an inhibitory mode (endo-ouabain) have been implicated in physiological processes including Na^+ excretion, heart failure, insulin responsiveness and hypertension. The effect occurs within the minute scale. Indirect and slow (hours to days scale) regulating mechanisms are mediated by hormones and growth factors. They regulate the quantity of active pumps by three different mechanisms: i. induction of the $(Na^+ + K^+)$-ATPase gene expresion, ii. post translational modifications of enzymes from a preexisting pool and iii. variation of the half life time of the enzyme.

Purification

Isolation of membrane bound enzymes is a tedious task. It took still 17 years after the discovery of the enzyme by Skou in 1957 before an appropriate method for purification of $(Na^+ + K^+)$-ATPase was developed (Jørgensen 1974a b, Esmann 1983). This method is nowadays the most widely used method for preparation of the enzyme, although some other approaches have been reported (Hokin et al 1973, Esmann et al 1979). In the method of Jørgensen (1974a) a homogenized suspension of the outer medulla of (rabbit) kidney is fractionated to obtain microsomes containing plasma membrane fragments. The contaminating proteins present in the microsomal preparations are removed by extraction with a concentration of sodium dodecyl sulfate, low enough to leave the $(Na^+ + K^+)$-ATPase unaffected in the lipid bilayer. After removal of the detergent by sucrose gradient zonal centrifuga-

tion, highly purified (Na^++K^+)-ATPase in membrane fragments is . obtained The resulting membrane segments exist of cup-shaped discs with a diameter of 100-600 nm (Maunsbach et al 1988a), containing (Na^++K^+)-ATPase with a frequency of 12,500 particles per μm^{-2} (Maunsbach et al 1988b).

This method is successful with kidney tissue because of the high density of pump molecules in this organ. The density of pump molecules varies from tissue to tissue. The number of pump molecules in red blood cells and in white blood cells have been estimated to be about 250 (Erdmann and Hasse 1975) and 30,000 (Boon et al 1984) per cell, respectively, whereas this number for kidney cells in the thick ascending limb of Henle is more than 40 million per cell (Mernissi and Doucet 1983).

The enzyme still contains equal amounts of lipid (on weight basis) surrounding the enzyme. The composition of the lipids surrounding the enzyme is strongly dependent on the species (Matsuda and Iwata 1986). The number of molecules in the lipid core surrounding the protein has been reported to be 50 phospholipid and 40 cholesterol molecules per dimer (Esmann et al 1980). More recently, a figure of 36 lipid molecules which can be accomodated around the intramembraneous section of (Na^++K^+)-ATPase $\alpha\beta$ protomer, based on structural data has been reported (Esmann et al 1988). The minimal number of lipid molecules surrounding the enzyme necessary for activity after partial delipidation has been determined to be 60 (Esmann et al 1979).

The specific activity ranges from 1000 to 2000 µmoles ATP split per hour per mg protein. This activity is completely lost upon removal of the accompanying lipids. Restoration of activity is obtained by addition of exogenous lipids, indicating an absolute requirement for lipids.

(H^++K^+)-ATPase from gastric parietal cells is usually isolated without the use of detergents (see however below). The procedure has been derived from methods originally reported by the groups of Forte (Forte et al 1974, Ray and Forte 1976 and Lee and Forte 1978) and Sachs (Saccomani et al 1977a) and has been modified by Schrijen (1981). Briefly, the collected scrapings of gastric mucosa of the fundic part of porcine stomach are homogenized and fractionated by centrifugation. Thereafter the pellets are separated on a sucrose-Ficoll gradient to obtain intact and broken gastric vesicles containing (H^++K^+)-ATPase. Further purification can be obtained by zonal electrophoresis (Walters and Bont 1979) and detergent treatment (Soumarmon et al 1983, Nandi et al 1987, Takaya et al 1987). Zonal electrophoresis mainly reduces the basal Mg^{2+}ATPase activity, a hydrolytic activity of the ATPase preparation which is not stimulated by K^+. This activity always accompanies the K^+-stimulated hydrolytic activity and cannot be totally removed by purification procedures (Saccomani et al 1977b). The K^+-stimulated activity of the enzyme ranges from 60 to 120 µmoles ATP split per hour per mg protein.

Structure

(Na^++K^+)-ATPase consists of two different subunits α and β. The α-subunit with an apparent molecular mass of about 100 kDa on SDS gel electrophoresis (and 94-121 kDa as determined by ultracentifugation (Esmann 1983)) is the catalytic subunit which can be phosphorylated by ATP and P_i and carries binding sites for ouabain and ATP.

The β-subunit is a glycoprotein containing about 50 carbohydrate units present in three trees coupled to asparagine residues (Esmann et al 1980) of 50 kDa on SDS page. The molecular weight determined by ultracentrifugation techniques appeared to be 36-42 kDa (Esmann 1983). The exact molecular masses of the protein part of the enzyme could be determined after elucidation of the primary structure of both subunits (see below). The catalytic function of the β subunit is unclear, but it is supposed to be involved in the insertion of the α subunit into the bilayer of the plasma membrane (Hiatt et al 1984). Based on the observation that reduction of the disulfide bond located in the β subunit of (Na^++K^+)-ATPase resulted in total inactivation of the enzyme, it was suggested that the β subunit must also be involved

in the maintainance of the enzyme structure (Kawamura and Noguchi 1988)

A third subunit different from α and β was noticed in brain tissue by Rivas et al (1972), who suggested that it could function as an extracellular receptor site for cardiac glycosides Later it was purified and designated the γ component of $(Na^+ + K^+)$-ATPase, although it is not present in tissues, like kidney (Forbush and Hoffman 1978, Reeves et al 1980) The γ subunit has been partially sequenced (Collins and Leszyk 1987) recently It appears to be an amphiphilic protein with the hydrophobic carboxyl terminal probably spanning the membrane The function of this subunit is hitherto unknown, although suggestions have been made, that it is involved in ouabain binding

The molar ratio of the α and β subunits in the holoenzyme is still subject of controversy Although there is consensus about the presence of both α and β subunits in the active protein complex, there is still uncertainty about the molecular weight of the holoenzyme. Based on the number of ligands bound to the enzyme ouabain, vanadate, ATP (high affinity) and inorganic phosphate incorporated in the enzyme, a minimum molecular weight value which varies from 175 to 270 kDa has been obtained (Jørgensen 1977, Smith et al 1980, Modzydlowski and Fortes 1981, Skou 1988) The molecular weight and composition of the minimal functional subunit, as determined by radiation inactivation and solubilisation techniques, differ strongly in reports of several research groups (for reviews see Nørby 1987, Reynolds 1988) Minimal numbers of monomers composing the functional holoenzyme vary from 1 to 4 (Esmann et al 1980, 1985, Jørgensen and Anderson 1986, Jensen and Ottolenghi, 1985, Askari 1987, Jensen and Nørby 1988, Dzhandzhungazyan and Modyanov 1985, Dzhandzhungazyan et al 1988, Cavieres 1988) The molecular weights obtained by these techniques vary from 170 to 379 kDa Based on molecular weight of about 112 kDa for the α and and 35 kDa for the β subunit (Shull et al 1985, 1986a) and a 1 1 ratio for the subunits (Peters et al 1981c) a monomer ($\alpha\beta$) or a dimer ($\alpha_2\beta_2$) are suggested by these figures It was reported that the monomer might correlate with phosphatase activity, whereas the dimer is involved in the hydrolytic and transport capacity (Ottolenghi 1979) An idea in line with the former is suggested by results of radiation inactivation experiments From these experiments it was observed that starting from the E_1 conformation increasingly larger particles seemed necessary to support partial reactions round the $(Na^+ + K^+)$-ATPase pump cycle. The α monomer seemed able to catalyse reversibly the reactions leading to E_2P A particle the size of α_2 or larger seemed necessary to obtain $(Na^+ + K^+)$-ATPase activity at 1 mM ATP Particles of intermediate size could support K^+-dependent hydrolysis by $(Na^+ + K^+)$-ATPase at 10 μM ATP (Cavieres 1988) Monomers and dimers have been observed with negative staining and freeze fracture electron microscopy, respectively (Maunsbach et al 1988a) Opposing models resulted from mechanical studies the single site model (Smith et al 1980, Moczydlowski and Fortes 1981) and the flip flop model of cooperating subunits (Stein et al 1973, Scheiner-Bobis et al 1988), corresponding to a monomer and dimer model, respectively

At this moment an unequivocal answer to the question whether ion transport systems work as functionally independent monomers or as oligomers is lacking The idea which has emerged is that for several partial reactions of the enzymes, the monomer seems to be sufficient, whereas for others the interaction between subunits is required

Depending on species and organ, one or two isoforms of the alpha subunit have been isolated alpha and alpha(+) (Sweadner 1979) The α(+) has been isolated from brain, muscle and fat tissue The two isoforms differ in ouabain sensitivi ty (Sweadner 1979), Na^+ affinity (Lytton 1985a) and N-terminal amino acid sequence (Lytton 1985b) Recently a third type of α subunit in rat brain (alpha III) was suggested from cDNA clon ing (Hara et al 1987) With molecular biological techniques all three isoforms of the α subunit have been identified up till now (Shull et al 1986b, Sweadner 1989)

$(Na^+ + K^+)$-ATPase occurs in two predomi nant conformational states the so called E_1 con

formation is predominant in presence of Na^+ and the E_2 in presence of K^+. The E_1 conformation in absence of Na^+ is also induced by ATP (Beaugé and Glynn 1980, Jørgensen and Karlish 1980), high pH (Skou and Esmann 1980) and high ionic strength (Karlish 1988). The E_2 in the absence of K^+ is induced by ouabain, phosphate (Karlish 1980), vanadate (Karlish and Beaugé 1979), low pH (Skou and Esmann 1980) and low ionic strength (Karlish 1988).

With the aid of physical techniques like infrared spectroscopy (Chetverin and Brazhnikov 1985) circular dichroism (Gresalfi and Wallace 1984, Hastings et al 1986) and Raman spectroscopy (Ovchinikov et al 1988) some details about the secondary structure of $(Na^+{+}K^+)$-ATPase have become clear. $(Na^+{+}K^+)$-ATPase contains a roughly equal mixture of α helical, β-sheet and random coil structures. Some controversy exists on the amount of changes from α-helix to β sheet involved in the conversion of E_1 to E_2. Gresalfi and Wallace (1984) reported a change of maximal 7%, whereas Chetverin and Brazhnikov (1985) could not detect a change of more than 2%. The latter result was confirmed by Hastings et al. (1986). With Fourier transform infrared spectroscopy only a minimal change in the secondary structure of $(H^+{+}K^+)$-ATPase was observed during the conformational change from E_1 to E_2 (Mitchell et al 1988). The latter observation also precludes a gross change in the secondary structure of $(H^+{+}K^+)$-ATPase.

The conversion of the different conformational states of the enzyme can also be monitored by fluorescence techniques. For $(Na^+{+}K^+)$-ATPase intrinsic tryptophan fluoresence (Karlish and Yates 1978), or extrinsic fluorescent probes like fluorescein isothiocyanate (Karlish 1980), formycin triphosphate (Karlish and Yates 1978) and eosin (Skou and Esmann 1981) have been used for this purpose. For $(H^+{+}K^+)$-ATPase fluorescein isothiocyanate (Jackson et al 1983) and eosin (Helmich de Jong et al 1986) have been used as probes for the conformational state of the enzyme. The two conformational states E_1 and E_2 can also be discriminatated on SDS gels after tryptic digestion of $(Na^+{+}K^+)$-ATPase (Jørgensen 1975) and $(H^+{+}K^+)$-ATPase (Helmich-de Jong et al 1987).

An idea about the 3-dimensional structure of $(Na^+{+}K^+)$-ATPase is obtained by the (Fourier) analysis of 2-dimensional crystals of $(Na^+{+}K^+)$-ATPase (Skriver et al 1981, Jørgensen et al 1982, Ovchinikov et al 1985, Skriver et al, 1985, Mohraz et al 1987) and $(H^+{+}K^+)$-ATPase (Rabon et al 1986). The unit cell of the crystals of $(Na^+{+}K^+)$-ATPase has the following parameters: a=66 Å b=118 Å and γ=108° and contains two protein density maxima (Demin et al 1988). A structure consisting of two symmetric rod-like structures, probably the α and β subunit, pertruding the membrane with a height of 10 nm perpendicular to the membrane. From crystallisation studies it was deduced that this structure emerges from the membrane 2 nm at the extracellular side and 4 nm at the cytoplasmic side.

Crystallisation of membrane enzymes in a 3-dimensional lattice could give worthful information on the structure of the enzyme, but appears to be difficult for intrinsic proteins. No successful crystallisation of $(Na^+{+}K^+)$-ATPase has been carried out so far.

The major part of information about the structure, however, is obtained by the determination of the primary structure by molecular biological approaches. The primary structure of the α and β subunits of $(Na^+{+}K^+)$-ATPase has been elucidated for several organs of different species: sheep kidney α (Shull et al 1985) and β subunit (Shull et al 1986a), electric organ of Torpedo californica α (Kawakami et al 1986) and β (Noguchi et al 1986) and pig kidney α (Ovchinikov et al 1986). The derived molecular mass of these sequences was 112 and 35 kDa for the protein part of the α and β subunit, respectively.

Uncertanties about the exact composition of the holoenzyme of $(H^+{+}K^+)$-ATPase also exist. The molecular weight of the protein part of the catalytic subunit of $(H^+{+}K^+)$-ATPase is exactly known after molecular cloning of the α subunit by Shull and Lingrel (1986). The enzyme consists of one or more subunits with an apparent molecular mass of about 100 kDa on SDS gel. The reported molecular mass of the holoenzyme,

however, ranges from 270 to 440 kDa. These masses are obtained by different techniques, among which radiation inactivation (Saccomani et al 1981, Schrijen et al 1983), gel filtration and gradient centrifugation (Soumarmon et al 1983, 1986). Recently a target size of 92-146 kDa was reported (Rabon et al 1988) for the destruction of the catalytic protein and the phosphoenzyme by radiation inactivation measurements. The latter size must represent a monomeric structure with possibly a second significantly smaller peptide.

The number of subunits is not clear neither from stoichiometry nor from ligand binding studies. For fluorescein isothiocyanate 1.5 nmol per mg protein has been reported (Jackson et al 1983), whereas, for vanadate binding and inorganic phosphate binding values of 2.8 ± 0.3 (Faller et al 1983) and 2.6 ± 0.1 (Jackson and Saccomani 1984) have been found. ATP binding with the labeled photoaffinity analogue N_3-ATP yielded 3.5 nmol ^{32}P incorporated per mg protein. Since of the latter amount only 50% was K^+ and hydroxylamine sensitive, it has been interpreted that 50% of the incorporated ^{32}P was bound ATP and the other 50% derived from phosphorylated enzyme (Faller et al 1982). Vanadate binds to the enzyme with two different affinities. Upon binding of 1.5 nmol vanadate per mg protein, half of the enzyme y activit was lost. From the above findings it might be tentatively concluded that 1.5 nmol per mg represents one site from the holoenzyme and that further binding must occupy a second site on the enzyme. Together with the uncertainties in the protein determination these findings make it difficult to determine the number of subunits of the holoenzyme. The minimal number of subunits for the fully active enzyme complex is therefore uncertain, but probably close to 3 or 4.

Another point of discussion is the homogeneity of the subunits. From tryptic digestion experiments (Saccomani et al 1979b) and isoelectric focussing the presence of three different subunits was concluded (Smolka et al 1983). In contrast to these results, however, Peters et al (1981a) found no clues for heterogeneity of the 100 kDa subunits by using several techniques (but no isoelectric focussing). Recently a second glycoprotein, distinct from the 100 kDa subunit with a molecular weight of 60-80 kDa has been observed in preparations of $(H^+{+}K^+)$-ATPase (Munson et al 1989). A monoclonal antibody has been developed that recognizes the latter glycoprotein on western blots from both rabbit and hog (Chow et al 1989). Endoglycosidase F treatment of this heavily glycosilated protein produced a core protein of 34 kDa (Okamoto et al 1989). This deglycosilated band is also recognized by the above mentioned antibody. It has been speculated that the calytic subunit of $(H^+{+}K^+)$-ATPase may be associated with a glycoprotein which has been putatively coined β-subunit, because of the resemblance with the β-subunit of $(Na^+{+}K^+)$-ATPase.

The primary structure of the catalytic subunits of $(H^+{+}K^+)$-ATPase of rat (Shull and Lingrell 1986) and pig (Maeda et al. 1988) have been determined through their complementary cDNA's. From hydropathy analysis (Kyte and Doolittle 1982) hydrophobic regions in the primary structure which might represent transmembrane domains have been derived. For the β subunit of $(Na^+{+}K^+)$-ATPase one such segment has been derived (Shull et al 1986b) with the glycosylated part at the extracellular side (Farley et al 1986) while six, seven or eight of these segments have been proposed for the alpha subunit of $(Na^+{+}K^+)$-ATPase (Shull et al 1986b, Noguchi et al 1986, Ovchinkov 1987, Ovchinikov et al 1987a, 1988). For $(H^+{+}K^+)$-ATPase seven to nine membrane spanning domains have been proposed (Shull and Lingrell 1986, Maeda et al 1986). By the computational method based on hydropathy plots, the exact number of membrane spanning domains could not be obtained. Determination of the sidedness of extramembraneous domains with the aid of immunochemical techniques (Kyte et al 1987, Ovchinikov et al 1988a b, Ohta et al 1988) and controlled proteolysis with specific chemical labeling (Jørgensen et al 1983) led to the conclusion that most of the protein of $(Na^+{+}K^+)$-ATPase including the N-terminal part would be present intracellularly, but on the location of the C-terminal parts of $(Na^+{+}K^+)$-ATPase various investigators disagree. Additional information about the structural arangement of the

transmembrane loops can be obtained by the use of antibodies against synthetic peptides which match the primary sequence of the ATPase (Rowe et al 1988). The assignment of luminal, transmembrane and cytoplasmic domains of the C-terminal region of $(H^+ + K^+)$-ATPase is also uncertain, whereas in the N-terminal region of the $(H^+ + K^+)$-ATPase the four major hydrophobic can be well deduced from hydropathy plots and assignments can be made since there is a large homology with $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase from sarcoplasmic reticulum (Serano et al 1986).

Cation binding sites

Three cytoplasmic binding sites for Na^+ (Garay and Garrahan 1973, Kaniike et al 1976, Yamaguchi and Tonomura 1980, Matsui 1983) showing positive cooperativity are responsible for the stimulation of the phosphorylation reaction of $(Na^+ + K^+)$-ATPase as well as the Na^+-ATPase activity (Robinson 1977, Flashner and Robinson 1979). Other observations are indicative for more cytoplasmic Na^+ binding sites. Fukushima (1987) proposed that the site where Na^+ inhibits dephosphorylation is located at the cytoplasmic side of the enzyme. Nørby et al (1983) observed inhibition of Na^+ on the E_1 to E_2 conformational change at sites which are probably cytoplasmic. Na^+ at low affinity binding sites can stimulate ADP dependent as well as independent dephosphorylation (Hara and Nakao 1981). A high affinity Na^+ binding site has been demonstrated by Cavieres and Ellory (1975).

Other sites with lower affinity ($K_{0.5}$, 24-40 mM) also activating the phosphorylation reaction have been described too (Foster and Ahmed 1976). The localization of the latter sites is unknown, but the findings that external Na^+-sites stimulate Na^+-ATPase, uncoupled Na^+ flux, ADP/ATP exchange and Na^+/Na^+ exchange (Glynn and Karlish 1976, Blostein 1983, Kaplan 1983) suggest that the sites which activate the phosphorylation reaction might be extracellular as well. They might represent the sites where Na^+ is discharged from the extracellular side of the enzyme. With reconstituted $(Na^+ + K^+)$-ATPase Cornelius and Skou (1987) have shown that extracellular Na^+ can take over the role of extracellular K^+ in dephosphorylation of the E_2P.

Three different classes of K^+ binding sites have been described and designated α, β and γ (Robinson 1983a). Extracellular K^+ sites (β) with high affinity ($K_{0.5}$, 0.1 mM) are necessary for the activation of the $(Na^+ + K^+)$-ATPase by speeding up the rate of the dephosphorylation reaction of the E_2P conformation (Robinson 1975, Cornelius and Skou 1987). Cytoplasmic K^+ sites (α) with moderate affinity ($K_{0.5}$, 2 mM) play a role in the K^+-phosphatase reaction (Drapeau and Blostein 1980). Cornelius and Skou (1987) describe a low affinity effect of cytosolic K^+ on the ATP affinity. Similar to the effect of high affinity extracellular K^+ the ATP curve becomes biphasic at high (> 50 mM) cytoplasmic K^+. Karlish and Pick (1981) also described cytoplasmic K^+ binding sites. The α sites correspond to the cytoplasmic sites where K^+ is discharged from the enzyme and from which site they might become occluded by a backward reaction of the pump.

The third class (γ) of K^+ sites are involved in reversal of the pump and act with low affinity at the cytoplasmic side (Robinson et al 1977).

The question whether Na^+ and K^+ sites are identical i.e. whether the transport of the two cations occurs via the same route is still not answered (Shani-Sekler et al 1988). Neither has it been elucidated whether all binding sites are directly involved in transport phenomena or whether they have allosteric properties as well (Beaugé and DiPolo 1979, Beaugé and Glynn 1979a, Blostein and Chu 1979, Blostein 1979, Karlish and Stein 1985, Apell and Marcus 1986, Beaugé and Campos 1986, Yoda and Yoda 1988).

Mg^{2+} together with ATP forms the substrate of the ATPase and is therefore supposed to act at the cytoplasmic side of the membrane. Yet Mg^{2+} and also Mn^{2+} induce the E_2 conformation (Robinson 1981, 1983). The sidedness of this effect is not clear yet.

Besides known K^+-binding sites (Stewart et al 1981) binding sites for Na^+ can be deduced from experiments in which a stimulation of the

enzyme activity by Na^+ was observed at alkaline pH (Ray and Nandi 1985)

Na^+-binding sites have also been postulated for (H^++K^+)-ATPase from experiments with reconstituted enzyme (Rabon et al 1985) in which Na^+ dependent (passive) Rb^+ transport across the membrane in two directions was observed Recently this idea was confirmed by demonstration of ATP dependent Na^+ transport by (H^++K^+)-ATPase in gastric vesicles at alkaline pH (Polvani et al 1989, Ditmars et al 1989) The experiments suggest that Na^+ binding competes with H^+ at the cytoplasmic side and with K^+ at the extracellular side.

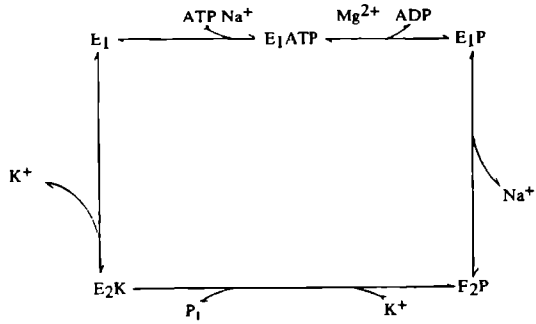
Reaction cycle

Both (Na^++K^+)-ATPase and (H^++K^+)-ATPase belong together with Ca^{2+}-ATPase and some H^+-ATPases to the "P"-type ATPases (Pedersen and Carafoli 1987) or E_1-E_2 ATPases (Racker 1985) This classification is based on the phosphorylated intermediates which they have in common in their reaction cycles and the existence of at least two conformational states in the reaction cycles defined E_1 and E_2

The reaction cycle of this type of transport enzymes was originally described by Post et al (1969, 1972) and Albers et al (1968) for (Na^++K^+) ATPase The scheme has later been modified by Karlish et al (1978) The so called Post-Albers scheme is the most widely used, although alternative schemes have been proposed a simultaneous model by Levitt (1980), a bicyclic by Plesner et al (1981a,b 1985) and a non-consecutive one by Skou (1985) The main argument for the latter two alternative reaction cycles was the observation that the rate of breakdown of E_1P is not fast enough for participation of E_1P in the overall cycle Some authors therefore proposed that the E_1P is part of the Na^+ cycle but not of the Na^+-K^+ cycle (Plesner et al 1981) Other groups, however still claim the E_1P to be competent in the allover reaction cycle in presence of Na^+ and K^+ (Froehlich et al 1983, Mårdh 1975 a,b, Robinson 1988, Hobbs et al 1985 and Steinberg and Karlish 1989)

In the Post-Albers scheme the enzyme goes through a series of consecutive reaction steps which will be described here briefly for (Na^++K^+)-ATPase (see scheme 1)

The first step in the reaction cycle of (Na^++K^+)-ATPase is the random binding of Na^+ and ATP The affinity for Na^+ of the E_1 conformation is high· 0 19-1 5 mM (Garay and Garrahan 1973, Robinson 1977, Nørby and Jensen 1971, Hegyvary and Post 1971) in the absence of K^+ and 6-24 mM in the presence of K^+ The range of the latter values is dependent on the ATP concentration (Swann and Albers 1980) ATP binds in the absence of K^+ to the enzyme in the E_1 conformational state also with high affinity K_d= 0 1-0 2 μM (Glynn and Karlish 1976, Robinson and Flashner 1979, Glynn 1985, Rossi and Garrahan 1985, Nørby 1983, Nørby and Jensen 1971, Hegyvary and Post 1971, Cornelius and Skou 1987)



Scheme 1
Minimal components involved in the (Na^++K^+)-ATPase pumping cycle.

In the presence of K^+ high (1 μM) and low (100 μM) affinity sites for ATP have been reported (Robinson 1983b) The K_d for the high affinity site is similar to the value in presence of only Na^+ and absence of K^+ This effect is exerted by extracellular and cytosolic K^+ with high and low affinity respectively (Cornelius and Skou 1987)

Lys 501 has been proposed as the binding site for the adenosine ring of ATP (Shull et al 1985,

Davis and Robinson 1988) but asp 710, cys 656 (Ovchinikov et al 1987b) and lys 719 (Ohta et al 1986) have also been claimed as possible candidates (Ball and Friedman 1987).

For each ATP molecule 3 Na^+ ions are bound to the enzyme (Mårdh and Post 1977, Yamaguchi and Tonomura 1979). In the presence of micromolar concentrations of Mg^{2+} which can be replaced by Mn^{2+} (Robinson 1981, Campos and Beaugé 1988), Co^{2+} (Richards 1987) and Pb^{2+} (Swarts et al 1987) but not by Ca^{2+} (Robinson 1985) ATP is able to phosphorylate the enzyme (Klodos and Skou 1977). The γ-phosphate group of ATP is hereby transferred and covalently bound to an aspartyl (369) residue of the enzyme (Grisham and Mildvan 1974, Degani et al 1974, Nishigaki et al 1974). The first phosphointermediate formed is the high-energy intermediate E_1P. This intermediate undergoes a conformational conversion to the low-energy phosphointermediate E_2P via the recently postulated E*P form (Nørby et al 1983, Klodos and Nørby 1987). The idea of the existence of more conformational different E-P forms is also demonstrated by Tanaguchi et al (1988) by phosphorylation with acetyl phosphate of the enzyme labeled with fluorescent probes.

The high energy form E_1P is sensitive to ADP (dephosphorylation rate is increased in the presence of ADP, but this intermediate is not hydrolyzed by water), but insensitive to K^+. The E_2P is insensitive to ADP, but K^+-sensitive (hydrolyzed by water), whereas the E*P form is sensitive to both K^+ and ADP. Dephosphorylation of E_1P in the presence of ADP is actually the back reaction of the phosphorylation reaction. It leads to formation of ATP and can be measured as the ADP-ATP exchange reaction.

The acid-stable phosphointermediates are sensitive to hydroxylamine, indicating that indeed a mixed anhydride is formed with both $(H^+{+}K^+)$-ATPase (Ray and Forte 1976) and $(Na^+{+}K^+)$-ATPase (Schuurmans Stekhoven et al 1984). The conformational change of the different phosphointermediates of the enzyme is proposed to occur in parallel with the liberation of Na^+ from the enzyme at the extracellular low affinity sites. The conformational state of the E-P forms is dependent on the Na^+ and K^+ concentrations (Steinberg and Karlish 1989, Nørby and Klodos 1983). The affinity for Na^+ at the extracellular side is much lower than at the cytosolic side so that Na^+ can easily be released from the enzyme.

The most important novelty added to the Post-Albers scheme is the concept of occluded ions. During its translocation across the membrane Na^+ is occluded in the enzyme which implies the binding of Na^+ to intramembraneous sites of the enzyme (Glynn et al 1984, Esmann and Skou 1985). The enzyme in the E_2P conformation can bind K^+ with high affinity (0.1 mM Robinson 1977), which accelerates the hydrolysis of E_2P to $E_2 + P_i$. The E_2P form is the more sensitive phospointermediate with respect to hydrolysis. This higher sensitivity might be due to differences in hydrophobicity of the microenvironment of the active site as proposed by De Meis et al (1982, 1988) for Ca^{2+}-ATPase and confirmed for $(Na^+{+}K^+)$-ATPase by Andersen and Jørgensen (1985) and Robinson (1989).

The binding of K^+ to the E_2 conformation of the enzyme results in an occlusion of the ion (Beaugé and Glynn 1979b, Glynn and Richards 1982a, 1984, Glynn et al 1983, Forbush 1982a, 1987a, b). This term is used (as for Na^+) for the way of binding in which the ion is bound to sites in the transmembrane region of the enzyme and cannot readily exchange with ions in the medium. In the normal transport mode of the reaction cycle of $(Na^+{+}K^+)$-ATPase K^+ ions enter the E_2P from the extracellular side and become occluded via a high affinity binding site. K^+ can, however, also enter the occluded site via the cytoplasmic low affinity site (Beaugé and Glynn 1979a). The transport of the ion is concomitant with the conformational change of an E_2 state to an E_1 state of the enzyme. The affinity for K^+ of this E_1 conformation is much lower than the affinity of the E_2P and therefore K^+ is released from the enzyme. The E_2 to E_1 conformational change, which is the rate limiting step in the reaction cycle can be accelerated by binding of ATP to the enzyme with low affinity (Karlish and Yates 1978) and Mg^{2+} (Schuurmans Stekhoven and

Bonting 1981). Cornelius and Skou (1987) propose two alternative routes for the deocclusion of K^+. A slow ATP independent route and an ATP dependent route which can be accelerated by ATP. ATP exerts this effect with low affinity (K_m=0.45 mM). Nucleotides other than ATP appear to be poor substrates for (Na^++K^+)-ATPase: CTP is hydrolysed by (Na^++K^+)-ATPase but with a rate which is only 15% of that with ATP (Skou 1974a) and GTP is hydrolyzed with a very low rate by (Na^++K^+)-ATPase, but its hydrolysis is hardly stimulated by K^+. The main reason for this behaviour is probably the failure of these nucleotides to stimulate the E_2--->E_1 conformational change (Fu et al 1985, Robinson 1983c). Other nucleotides are not hydrolysed at all.

A concept based on proton accepting properties of the nucleotide might explain the cause of this failure (Boldyrev and Svinukhova 1982, Boldyrev et al 1984, 1988, Svinukhova and Boldyrev 1987). A shift in the poise of the equilibrium of E_1 and E_2 towards E_1 upon increasing the pH suggests that the E_1 conformation is the deprotonated form. This led Skou (1982) to the proposal that the conformational change from E_1 to E_2 occurred in parallel with a decrease in the pKa value of the enzyme. A possible candidate for deprotonation is the imidazole group of His 13 (Jørgensen and Collins 1986). ATP bound to the enzyme at the low affinity site could be the proton acceptor and thus could facillitate the conformational change from E_1 to E_2. Boldyrev (1988) observed that in a series of adenosine derivatives with increasing pK_a values the effectiveness of stimulation of the enzyme activity decreased and argued that the proton accepting properties of the nucleotide indeed influenced the conformational change. The effect of acetylphosphate also fits in this concept. This substrate analogue can phosphorylate the enzyme, but is not able to drive the Na^+ K^+ exchange (Beaugé and Berberian 1984) probably due to its failure to drive the E_1 E_2 conformational change. The observation that the ATP-site specific reagent p-fluorosulfonyl benzoyl adenosine does not induce an E_1 conformation of the enzyme (Johnson et al 1986) also fits in the concept. Other ATP analogues like ADP and adenylyl imidophosphate stimulate (Na^++K^+)-ATPase activity in the presence of low concentrations of ATP which are too low to stimulate the conformational change (27 μM). These compounds are supposed to replace ATP in its function at the low affinity binding site (Suzuki et al 1987).

No congener of Na^+ can replace its function in the phosphorylation reaction (Post et al 1969), whereas stimulating (and inhibitory) effects of several amines with respect to the phosphorylation and dephosphorylation reaction have been described (Schuurmans Stekhoven et al 1985, 1986b, 1988, Fukushima 1987). A Na^+-like effect of H^+ in several reaction steps has been reported (Blostein 1985, Hara and Nakao 1986, Polvani and Blostein 1988). Hara and Nakao (1986) reported that H^+ is transported instead of Na^+ and Polvani and Blostein (1988) also reported K^+ like effects of H^+. The idea that H^+ also substitutes for Na^+ with respect to the phosphorylation reaction is challenged by Schuurmans Stekhoven et al (1986a). These authors state that not H^+, but rather the positively charged amine groups of the buffer stimulate the phosphorylation reaction by inhibition of the dephosphorylation step (Schuurmans Stekhoven et al 1986b).

The accelerating effect of K^+ on the dephosphorylation reaction and on the overall reaction is shared by several congeners: Tl^+> Rb^+= K^+> Cs^+= NH_4^+> Li^+ in order of decreasing affinity (Robinson 1977). A different order of effectiveness Rb^+< Tl^+< Cs^+< K^+ has been found with respect to the ATP stimulated deocclusion of Rb^+ (Forbush 1987b). Results of experiments with modified carboxyl groups suggest that the occluded Na^+ and K^+ ions are bound to the same carboxyl residues in a non-aqueous binding domain (Shani-Sekler et al 1988).

For (H^++K^+)-ATPase a similar Albers-Post scheme is proposed and adjusted by Wallmark et al (1980) and Stewart et al (1981) and quantified by Brzezinski et al (1988). For (H^++K^+)-ATPase also an aspartyl-P is found to be the phosphorylated intermediate (Walderhaug et al 1985). Some deviations from the characteristics of

(Na^++K^+)-ATPase described above have to be mentioned: i. the role of Na^+ is overtaken by H^+. ii. the substrate specificity is also strict, but the effectiveness is somewhat different: only CTP and GTP can replace ATP with 15 and 12% of the rate of ATP (Sachs et al 1978). iii. a slightly different order for the affinity of K^+ congeners is observed: Tl^+> K^+> Rb^+> NH_4^+> Cs^+ (Sachs et al 1976, Forte et al 1980). iv. K^+ inhibits the phosphorylation rate voltage dependently (Lorentzon et al 1988). v. although K^+ must formally be occluded during the translocation across the membrane, it is difficult to show an occluded enzyme form and direct stimulation of the deocclusion by nucleotides. The reason for this problem may be the poor time resolution of the method with which the occlusion is monitored. Since the rates of the active Rb^+ efflux was similar to that of the passive Rb^+/Rb^+ exchange (Rabon et al 1985), it may be doubted that the deocclusion of K^+ is rate limiting in the reaction cycle as it is in the (Na^++K^+)-ATPase reaction cycle. vi. the most important difference between the two ATPases lies in the stoichiometry of transported cations and ATP hydrolysed. Under normal conditions (Na^++K^+)-ATPase transports 3 Na^+ against 2 K^+ ions per ATP molecule split (Kaplan 1985), whereas (H^++K^+)-ATPase exchanges an equal number of K^+ against H^+ per hydrolysed ATP molecule in the opposite direction. Values of 2 H^+ per ATP have been reported in both native vesicles and reconstituted (H^++K^+)-ATPase (Skrabanja et al 1986, 1987). Under physiological conditions, however (with a pH gradient of six units), maximally one H^+ per split ATP molecule can be transported for thermodynamical reasons. This does not change the conclusion that in contrast to ion transport by (Na^++K^+)-ATPase which results in the net translocation of 1 charge per cycle, ion transport by (H^++K^+)-ATPase is electroneutral. This electroneutral behaviour does not necessarily mean that the two ion translocating steps of (H^++K^+)-ATPase are both electroneutral. Lorentzon et al (1988) have demonstrated recently that the (H^++K^+)-ATPase is sensitive to membrane potentials produced in gastric vesicles and that the K^+-translocating step was voltage dependent, indicating that this step was electrogenic.

Other partial reaction steps not associated with ion fluxes

Two partial reactions of (Na^++K^+)-ATPase and (H^++K^+)-ATPase have not been described here so far: the phosphorylation of the enzyme by inorganic phosphate (P_i) and the K^+-stimulated phosphatase (pNPPase) reaction.

The phosphorylation by P_i is formally the reverse of the dephosphorylation of the E_2P conformation: E_2P--->E_1 + P_i (Robinson and Flashner 1979, Askari and Huang 1983, Askari and Huang 1984). A K^+- and hydroxylamine sensitive phosphointermediate is formed. The phosphorylation reaction by P_i requires Mg^{2+} for both (Na^++K^+)-ATPase (Post et al 1975) and (H^++K^+)-ATPase (Jackson and Saccomani 1984). The divalent cation is probably bound to a site which is different from that involved in the phosphorylation reaction by ATP (Forbush 1987b). The K_m for P_i is 60 and 70 μM for (H^++K^+)-ATPase (Jackson and Saccomani 1984) and (Na^++K^+)-ATPase (Schuurmans Stekhoven et al 1980), respectively. The phosphorylation reaction of (Na^++K^+)-ATPase by P_i is inhibited by Na^+ (Post et al 1975, Schuurmans Stekhoven et al 1980). On the other hand P_i can also pH-dependently inhibit (Na^++K^+)-ATPase activity with low affinity (28 mM and 12 mM at pH 7.1 and 7.8 repectively) (Huang and Askari 1984).

The pNPPase activity is always copurified with either of the two enzymes and it has been shown that this hydrolytic activity is a partial reaction of the enzyme (Skou 1965, Schrijen et al 1983), although not correlated with transport. The reaction needs Mg^{2+} with low affinity (Robinson 1969) and is stimulated by K^+ (which to a certain extent can be replaced by Ca^{2+} (Vasallo and Post 1986)) and is inhibited by ATP and phosphate (Ljungström et al 1984, Ray and Nandi 1985, Robinson and Flashner 1979, Robinson et al 1983).In the case of (Na^++K^+)-ATPase the pNPPase activity is also inhibited by Na^+. Maximum activity is obtained in presence of 100 mM K^+, 20 mM Mg^{2+} and 10 mM substrate. At 37°C 14-18% of the turnover rate of the (Na^++K^+)-ATPase activity is reached (Skou

1974b). The pNPPase is supposed to be a hydrolytic reaction of the E_2 conformational state of the enzyme, with or without K^+ occluded (Robinson 1983a, Campos et al 1988, Berberian and Beaugé 1985). From experiments with inverted erythrocyte ghosts it was concluded, that K^+ stimulates this reaction at the cytoplasmic side with low affinity, whereas extracellular K^+ is without effect (Drapeau and Blostein 1980). The effect is supposed to be exerted by K^+ in the occluded state (Robinson 1983a). K^+ sites located at different sides of the membrane, however, have been reported to be stimulating with respect to the phosphatase reaction for $(H^+{+}K^+)$-ATPase (Ray and Nandi 1986) and $(Na^+{+}K^+)$-ATPase (Nandi et al 1988).

Another phosphatase activity of $(Na^+{+}K^+)$-ATPase which uses para nitrophenyl phosphate as substrate in the presence of Na^+ and ATP needs low extracellular K^+ (Skou 1974a). This latter activity has a lower turnover than the phosphatase reaction stimulated by low affinity cytoplasmic K^+, needs K^+ at both sides of the membrane (Drapeau and Blostein 1980) and is stimulated by low concentrations of ATP and oligomycine. Since oligomycine occludes Na^+ in the E_1 conformation (Hobbs et al 1983, Esmann and Skou 1985) it is suggested that this phosphatase activity is due to an effect of K^+ on the extracellular sites on an E_1 conformation of the enzyme with Na^+ occluded (Skou 1988).

Lipid involvement

$(Na^+{+}K^+)$-ATPase and $(H^+{+}K^+)$-ATPase require a hydrophobic environment for their function. In the native situation they are embedded in a lipid matrix consisting mainly of phospholipids and cholesterol. The requirement for specific lipid components with respect to the optimal activity of the enzyme has been subject for studies during several years.

Delipidation by detergents causes inactivation of the enzyme. Reactivation is obtained by addition of exogenous lipids. Early work suggests a specific requirement of $(Na^+{+}K^+)$-ATPase for the negatively charged phospholipids: phosphatidylserine, phosphatidylglycerol, phosphatidic acid and phosphatidylinositol (Kimelberg 1976, Kimelberg and Papahadjopoulos 1972, Tanaguchi and Iida 1971, Hokin and Hexum 1972, Wheeler at al 1975, Palatini et al 1977, Walker and Wheeler 1975, Mandersloot et al 1978). This suggestion is opposed by the observation that enzymatic conversion of the negatively charged phospholipids causes only a minor loss of activity (De Pont et al 1978).

Experiments with $(Na^+{+}K^+)$-ATPase incorporated in lipid vesicles of known composition showed that either phosphatidylethanolamine (Racker 1985) or phosphatidylcholine (Hilden and Hokin 1976) were suitable to reconstitute activity of the ATPase. For optimal recovery of the activity of the reconstituted enzyme phosphatidylinositol and phosphatidylethanolamine have been reported to be necessary (Cornelius and Skou 1984).

The role of cholesterol is still under discussion: some authors claim a specific requirement of $(Na^+{+}K^+)$-ATPase for cholesterol (Noguchi et al 1971, Järnefelt 1972, Seiler and Fiehn 1976), while others conclude it to be non-essential (Roelofsen and Van Deenen 1966, 1973, Wheeler and and Isern De Caldentey 1980, Peters et al 1981b, Yeagle 1983). Effects of cholesterol on the composition of the phosphorylated intermediates have been reported (Yoda and Yoda 1987): high cholesterol contents in the vesicles resulted in a lower $E_2P{:}EP_{total}$ ratio at room temperature.

The effects of the acyl chains of phospholipids have also been studied extensively. An optimal reactivation of delipidated enzyme was obtained with phospholipids containing fatty acids with a chain length between 16 and 21 carbon atoms (Johannsson et al 1981), whereas it was reported (Kimelberg and Papahadjopoulos 1972, Palatini et al 1977, Ottolenghi 1981) that increasing fluidity of the membrane increases reactivation. Kimelberg and Papahadjopoulos (1974) observed a reactivation with increasing effectiveness in the series 18:0<16:0<14.0<18:1. Increase of the number of unsaturated C-C bonds in the acyl chains of the phospholipids surrounding the $(Na^+{+}K^+)$-ATPase increases the hydrolyt-

ic activity of the enzyme (Kimelberg and Papahadjopoulos 1974, Walker and Wheeler 1975).

The transport capacity of the proteoliposomes containing (Na^++K^+)-ATPase is dependent on the chain length of the mono-unsaturated alkyl chains: chains longer than 16 C atoms stimulate high pumping activities, whereas, shorter chains cannot restore pump activity. Moreover increase of the unsaturation of the acyl chains reduces the hydrolytic activity and pumping rate (Marcus et al 1986). These results agree with those observed by Johanson et al (1981) with respect to the effect of the chain length of the acyl chains and their degree of saturation opn the hydrolytic activity of the enzyme. Abeywardena et al (1983) reported a decrease of activity with increasing length and full reactivation with dioleoylphosphatidylcholine in reconstituted (Na^++K^+)-ATPase.

The requirement of certain phospholipids for the activity of (H^++K^+)-ATPase has been investigated using different specific phospholipases. Hydrolysis of lipids in intact vesicles with phospholipase A2 reduced the K^+-stimulated hydrolytic activity which could most effectively be restored by addition of phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine (Saccomani et al 1979a). Treatment of (H^++K^+)-ATPase with phospholipase C resulted in inactivation in parallel with loss of phospholipids (Schrijen et al 1981), but no specific phospholipid requirement could be observed upon relipidation.

Inhibitors

A common characteristic of the P-type ATPases is the inhibitory effect of vanadate, a transition state analogue of phosphate (resembling it in its leaving group properties). The vanadate ion is supposed to bind to the phosphate binding site hereby preventing the phosphorylation of the enzyme (Cantley et al 1978).

Specific inhibitors for (Na^++K^+)-ATPase are represented by the group of cardiac glycosides. The water soluble compound ouabain (g-strophantin) which binds with high affinity at the extracellular side of the enzyme is the most widely used representative of this group. The more hydrophobic compound digoxin can penetrate membranes and has advantages, when used with reconstituted systems, because it can also inhibit inside-out oriented (Na^++K^+)-ATPase molecules (Rey et al 1987, 1988, Anner et al 1988).

The search for specific inhibitors of (H^++K^+)-ATPase is an important goal in ulcer research. So far two classes of specific inhibitors of (H^++K^+)-ATPase have been extensively studied: the substituted benzimidazole compounds with omeprazole as the main representative (Larsson et al 1983, Lind et al 1983 for an overview see Lindberg et al 1987) and substituted pyridyl 1,2a imidazole compounds (sulphinylbenzimidazoles) of which the compound SCH 28080 is the best studied one (Long et al 1983, Ene et al 1982). Another newly synthesized compound of the first mentioned class has been described recently (Sigrist-Nelson et al 1986, 1987). Some other inhibitors on gastric acid secretion like fenoctimine (Reenstra et al 1986) and nolinium bromide (Nandi et al 1983, Nandi and Ray 1987) have been examined on their direct action on (H^++K^+)-ATPase, but appear to be less effective and/or less specific than the inhibitors of the two classes mentioned above.

The direct action of omeprazole on the (H^++K^+)-ATPase, as mechanism explaining its inhibitory effect on acid secretion, was proven by observations of Wallmark et al (1985). Inhibition of the hydrolytic activity of (H^++K^+)-ATPase appeared to occur in parallel with inhibition of acid secretion in rat (Wallmark et al 1985). The selectivity for (H^++K^+)-ATPase of this class of inhibitors is mainly based on the requirement of protonation for activation of the inhibitor (Wallmark et al 1984, Im et al 1985). Omeprazole is a weak base with a pKa value of 4. In the unprotonated form it is lipophilic enough to penetrate lipid bilayers. The latter two properties cause the accumulation of this compound in acid compartments (e.g. in vitro in gastric membrane vesicles). Protonation of the inhibitor in the acid compartment induces a cascade of rearangements leading to the active derivative: a sulfenamide (Lindberg

et al. 1986). This active inhibitor compound reacts with essential sulfhydryl groups of the enzyme resulting in inhibition of the enzyme (Lorentzon et al 1985). Inhibition is complete when one to two inhibitor molecules are bound per phosphoenzyme (Lorentzon et al 1987). In vivo the tubulovesicular system in the parietal cell is acidified by the proton pump and forms a suitable environment for protonation of the drug. The site of acidification is in close proximity of $(H^+ + K^+)$-ATPase. The specificity of the inhibition by omeprazole is predominantly based on this acidification since the drug inhibits also $(Na^+ + K^+)$-ATPase, although with low affinity: the IC_{50} values are 19 μM and 186 μM at pH 6.1 and 7.4 respectively, whereas these values are respectively 5.2 μM and 36 μM for $(H^+ + K^+)$-ATPase (Keeling et al 1985). After transient acidification of the drug the IC_{50} for $(Na^+ + K^+)$-ATPase becomes 19 μM. which is not much different from 5.2 μM under the same conditions for $(H^+ + K^+)$-ATPase (Keeling et al 1985). Inhibition by omeprazole is irreversible, which is in contrast to the reversible inhibition by the second class of specific $(H^+ + K^+)$-ATPase inhibitors.

The protonable amine SCH 28080 (2-methyl-8-[phenyl methoxy] imidazo-1 (1-2-a) pyrine-3-acetonitrile) is an even more selective inhibitor of $(H^+ + K^+)$-ATPase which is shown by its IC_{50} value of 0.024-0.8 μM (depending on the K^+-concentration) compared to the IC_{50} value for $(Na^+ + K^+)$-ATPase, which is three orders of magnitude higher (Scott et al 1987, Wallmark et al 1987). SCH 28080 is also the more specific representative of its class of inhibitors (Beil et al 1987). The inhibition of $(H^+ + K^+)$-ATPase activity by SCH 28080 and analogues is competitive with K^+ and uncompetitive with respect to ATP (Wallmark et al 1987). The inhibitor probably interacts with the E_2 conformational state of the enzyme, by binding to the luminal K^+ binding site (Wallmark 1987). For this reason derivatives have been synthesized to probe the K^+ binding site (Munson and Sachs 1988).

Reconstitution of transport ATPases

During many years the properties of transport enzymes have been studied using preparations of purified enzymes. These enzyme preparations usually consist of the protein under study embedded in a fragment of the native membrane. The advantage of this preparation is obvious, the enzyme retains its activity and can be studied as an isolated entity without interference of other membrane proteins. The main disadvantage of this system, however, is the loss of compartmentalisation. For its major function, active movement of ions across the membrane, the ATPase requires two compartments. In membrane fragments the transport properties have been lost.

In other preparations, in which the compartimentalization is maintained (like cells or native vesicles, containing transport enzymes), the measurement of transport is possible, but the disadvantage of these systems is the presence of other proteins, which cannot be removed without disturbing the vesicular integrity. These proteins may disturb the measurements or obscure interpretations. For transport studies with $(Na^+ + K^+)$-ATPase, erythrocytes or erythrocyte ghosts and microsomal vesicles and for $(H^+ + K^+)$-ATPase gastric membrane vesicles have been used.

It would be desirable to study transport phenomena with highly purified enzyme facing two different compartments with its intra- and extracellular side, respectively.

Providing this compartmentalisation to the purified fragmented enzyme is here defined as reconstitution (for Reviews see Hokin 1981 and Madden 1986). It was the reconstituted system, in which it was proven that the purified $(Na^+ + K^+)$-ATPase is indeed the Na^+ pump. Arguments were provided by Hokin (1979) by comparison of the characteristics of reconstituted purified $(Na^+ + K^+)$-ATPase and the Na^+ and K^+ pumps in erythrocytes and squid axon. The stoichiometry of Na^+ and K^+ transport, ouabain inhibition, ATP hydrolysis, substrate specificity and vanadate inhibition showed good correlation. Reconstitution of membrane bound enzymes can

also give a conclusive answer in the discussion on the minimal subunit composition, nesessary for a functional Na^+-K^+ pump. The problem, however, is the inactivation of enzyme caused by the solubilisation step, necessary for the separation of the subunits. Reconstitution also provides the possibility to study the electrogenicity of different steps in the reaction cycle of transport ATPases.

Reconstitution in lipid vesicles

For the successful reconstitution of a membrane bound enzyme into a liposome several manipulations with the enzyme and lipids have to be carried out. In general these steps include solubilization of the enzyme, formation of liposomes from the lipids, some manipulations to stimulate the incorporation of the protein into the bilayer and finally removal of the detergent (Eytan 1982). The final product of these steps, a protein containing lipid vesicle, is called proteoliposome. Several properties of these proteoliposomes depend mainly on the method used for preparation and the lipid composition of the liposomes. For $(Na^+ + K^+)$-ATPase several methods have been used for reconstitution of the enzyme in functional proteoliposomes.

i. Cholate dialysis method.
The cholate dialysis method was developed originally by Kagawa and Racker (1971) for the study of components of the mitochondrial oxidative phosphorylation and later for the reconstitution of Ca^{2+}-ATPase from sarcoplasmic reticulum (Racker 1972). This method was used in the first attempt to reconstitute $(Na^+ + K^+)$-ATPase by Goldin and Tong (1974). They incorporated canine $(Na^+ + K^+)$-ATPase into lipid vesicles by slow removal of cholate by means of dialysis. They obtained proteoliposomes with low transport activities and K^+:Na^+ ratios far below 2:3. A modified cholate dialysis method for reconstitution of dog kidney $(Na^+ + K^+)$-ATPase by Hilden and Hokin (1975) and Goldin (1977) yielded much better proteoliposomes showing a stoichiometry of 3:2:1 for Na^+:K^+-pumped:ATP hydrolyzed. The rate of hydrolysis, however, was still low in these proteoliposomes (less than 8 μmol P_i per h per mg protein). The cholate dialysis method was further elaborated by Anner et al (1977), Jørgensen and Anner (1979), Skriver et al (1980), Anner and Moosmayer (1981), Anner et al (1984) (for reviews see also Anner 1985a,b, 1988 and Goldin et al 1988). Anner et al (1977) found with this preparation a ratio of 3:2 for Na^+ and K^+ transport for $(Na^+ + K^+)$-ATPase form lamb kidney medulla, but 5:1 for dog kidney $(Na^+ + K^+)$-ATPase.

ii. Dilution method
In this method $(Na^+ + K^+)$-ATPase from electric eel has been solubilized by high concentrations of cholate (2%) in the presence of sonicated phospholipids. The concentration of the detergent was thereafter decreased at least 20-fold by dilution of the sample into the assay medium or by dilution in buffer followed by centrifugation. This method originally developed by Racker et al (1975) yields actively pumping proteoliposomes (45 μmol Na^+ pumped per h per mg protein) (Racker et al 1979).

iii. $C_{12}E_8$ removal
This method of reconstitution was reported by Cornelius and Skou (1984) for reconstitution of $(Na^+ + K^+)$-ATPase from the rectal gland of the spiny dogfish. By this method the enzyme is solubilized with the nonionic detergent $C_{12}E_8$ before addition to sonicated liposomes and this step is followed by removal of the detergent by means of Bio-Beads. Full recovery of the specific activity of the incorporated enzyme molecules has been obtained with a protein:lipid ratio of 1:20 or higher.

iv. Gel filtration method
Hog kidney aminopeptidase (Allen et al 1980) and the hydrophobic peptide of MN-glycoprotein from erythrocyte membrane (Hall and Brodbeck 1978) have been reconstituted into phosphatidylcholine liposomes by a detergent solubilization procedure which was followed by rapid removal of detergent by column chromotography during formation of the proteoliposomes. This method was later successfully applied for reconstitution of

$(Na^+ + K^+)$-ATPase of bovine brain with only 10% loss of activity (Abeywardena et al 1983). The detergent in this procedure was removed by Sephadex G50 chromatography.

A variation of this method has been described by Yoda et al (1984). In their procedure, $(Na^+ + K^+)$-ATPase from electric eel was solubilized with Chaps and the detergent was removed by gel filtration. These authors reported full recovery of enzyme activity.

v. Sonication method

A method in which no use was made of detergents, but of prolonged sonication is originally described by Racker (1973) for bacteriorhodopsin and later addapted for $(Na^+ + K^+)$-ATPase (Racker and Fisher 1975). A mixture of dried phospholipids and $(Na^+ + K^+)$-ATPase was sonicated during 30 min at 5°C. This method leads to only a minor incorporation of the enzyme in the formed liposomes. The specific activity of the incorporated molecules, however, is 10-20 times higher than with the cholate dialysis method.

vi. Freeze-thaw-sonication method

This method was originally described by Kasahara and Hinkle (1977) for reconstitution of the glucose transporter from human erythrocytes. This method in which no detergent is used (Hokin and Dixon 1979, Dixon and Hokin 1980) yields higher transport and hydrolysis rates than the cholate dialysis method. In this method a mixture of enzymes and preformed liposomes were frozen and thawed repeatedly before sonication. This method combined with partial solubilization of the enzyme appeared also to be suitable for the reconstitution of gastric $(H^+ + K^+)$-ATPase (Rabon et al 1985). The enzyme solubilized in either n-octylglucoside or cholate is mixed with a suspension of sonicated lipids. This mixture is frozen and thawed prior to sonication. The detergent is removed by gel filtration on a Sephadex G-50 column.

vii. Freeze-thaw method

A variation on the method of Kasahara and Hinkle (1977) was used by Karlish and Pick (1981) to reconstitute $(Na^+ + K^+)$-ATPase. These authors prepared the liposomes in the presence of cholate and added solubilized $(Na^+ + K^+)$-ATPase to the liposomes before the freezing and thawing step. The sonication step was left out of the procedure and the detergent was removed by gel filtration. A variation of this method was introduced in which the detergent $C_{12}E_8$ was removed by absorbance to Bio-Beads (Brotherus et al 1983).

Several parameters play a decisive role in the success of the reconstitution procedure. The choice of a suitable detergent for a specific enzyme is very important for the solubilization of the enzyme. Solubilization of $(Na^+ + K^+)$-ATPase usually occurs in parallel with reduction of the enzymatic activity (Helenius and Simons 1975, Powell and Cantley 1980, Esmann and Skou 1984, Esmann et al 1980, Esmann 1986, Ottolenghi et al 1986, Soumarmon et al 1986, Morohashi et al 1988 and for reviews see also Brotherus et al 1979 and Møller et al 1987). The extent of inactivation is influenced by the solubilization conditions like temperature, pH, ionic strength and the presence of specific cations.

Since the inactivating and denaturing effects often occur in the concentration range necessary for solubilization, the working range for detergents is very narrow and specific: the detergent treatment is a balance between a concentration high enough to solubilize but not as high as to inactivate the enzyme. The interaction between a detergent and a particular enzyme is very complex and the effects on the enzyme are not always well understood. The conditions for solubilization like pH, temperature, time, protein:detergent ratio and ionic strength of the medium are important parameters for successful solubilization and therefore for reconstitution methods in which solubli-zation is involved.

Other factors crucial for the quality of the reconstituted preparation are the lipid:protein ratio and the composition of the bilayer lipids. The polar headgroup of the lipids is important for the functionality of the reconstituted enzyme. Several phospholipid compositions have been reported to be suitable for reconstitution of $(Na^+ + K^+)$-ATPase (Racker 1985). The hydro-

phobic interaction of the protein with the lipids is mainly determined by the composition of the acyl chains of the phospholipids. The composition of the fatty acids in the bilayer, therefore plays an important role in the incorporation and functionality of the enzyme in the bilayer (Marcus et al 1986).

The above mentioned parameters might influence the orientation of the incorporated enzyme. Most preparations are randomly (50:50) oriented, but variation of the lipid composition, membrane charge, diameter of the vesicle, lipid:protein ratio and the reconstitution method may induce oriented incorporation of the enzyme (Baldwin et al 1980, Jones et al 1981, Cornelius and Skou 1984, McCormick and Johnstone 1986).

Reconstitution in or on a planar bilayer (BLM)

Planar phospholipid bilayers (more familiar: Black Lipid Membranes) have been used as tools to study passive properties of the biological membrane (Müller et al 1962). The system has the advantage above proteoliposomes that electric events can be measured directly. Not much later the BLM system appeared to be an appropriate instrument to study transmembrane carriers and channels (Müller et al 1969, Latorre et al 1984, 1989). Rhodopsin, the acetylcholine receptor, porin, Na^+- and Ca^{2+}-channels, the proton channel of proton translocating ATPases and later also ion pumps as the reaction centre from photosynthetic bacteria, cytochrome C oxidase, bacteriorhodopsin, $(Na^+{+}K^+)$-ATPase (Mironova et al 1986) and a bacterial H^+-ATPase (Hirata et al 1986) have been reconstituted successfully and studied with this method (for a review see Montal et al 1981). In most studies the proteins are supposed to be embedded in the bilayer or to span it from one compartment to the other.

Studies with bacteriorhodopsin, however, indicate that the enzyme was not incorporated in, as supposed by Dancshazy and Karvaly (1976), but rather attached to the bilayer. The observations that the steady-state photocurrent strongly increases in the presence of gramicidin A (Bamberg et al 1979) or a proton conductor (CCCP) (Herrmann and Rayfield 1978) argue against the idea that bacteriorhodopsin spans the planar bilayer. The most likely interpretation of these findings is that the purple membrane sheets are associated to the black lipid membrane in a preferential orientation (Herrmann and Rayfield 1978, Bamberg et al 1979). Since the cation permeability of the BLM is low, only a transient displacement photocurrent occurs in absence of proton conductors. In the presence of protonophores a steady state photocurrent can be observed.

This model was analyzed on the basis of an equivalent circuit consisting of a capacitance and resistance of the membrane sheets and the BLM in series (Herrmann and Rayfield 1978, Bamberg et al 1979, Fahr et al 1981, Bamberg et al 1984). A similar system in which the pump molecule is attached to the black lipid membrane has been developed by the group of Bamberg to study pump currents generated by cation transport ATPases (Fendler et al 1985,1986, Hartung et al 1987, Nagel et al 1987, Fendler et al 1987, 1988).

Objectives of this study

The main goal of this study is to elucidate some structural and mechanistic questions concerning $(H^+{+}K^+)$-ATPase and $(Na^+{+}K^+)$-ATPase with special attention focussed on the phosphorylation reaction. In order to obtain information on characteristics of the two enzymes which cannot be studied with fragmented enzyme part of this study was carried out with the two enzymes reconstituted in two different systems.

Transport ATPases reconstituted in lipid vesicle not only provide a system for measuring ion transport phenomena, but offer the possibility of the determination of sidedness of ligands on several parameters of enzyme activity. With reconstituted $(Na^+{+}K^+)$-ATPase the role of physiological important ligands in the phosphorylation reaction has been studied. Na^+ and K^+ not only bind to sites from where they can be transported, but also to allosteric sites (Robinson 1983a). From the

results of these experiments with reconstituted $(Na^+ + K^+)$-ATPase conclusions could be drawn about the location of binding sites for the cations and the possible functionality of these binding sites.

Effects of amine compounds mimicking the effects of Na^+ and K^+ with respect to the phosphorylation reaction are hitherto described for unsided preparations. Some substances showed both inhibitory and stimulatory effects on the phosphorylation reactions which hindered the interpretation of the results. By studying the sidedness of action of these ligands it was possible to elucidate their mechanism of action by geometric separation of inhibitory and stimulatory effects.

Since there is a very close interaction between the ATPase and the lipids of the bilayer surrounding it in the reconstituted vesicle, this system provides the possibility to study lipid-protein interactions. Strong dilution of the endogenous lipids surrounding the enzyme will occur because there is a rapid transversal exchange of lipids in the bilayer. By addition of relatively large quantities of exogenous lipids of known composition the dependency of the enzyme activities on this new lipid environment can be studied in reconstituted $(Na^+ + K^+)$-ATPase.

Electric properties of the reaction steps correlating with the phosphorylation reaction of $(H^+ + K^+)$-ATPase have been studied with this enzyme reconstituted on a planar bilayer. Although $(H^+ + K^+)$-ATPase is an electroneutral working transport enzyme, separation of the reaction steps revealed that the H^+ translocating step which is concomittant with the phosphorylation reaction is electrogenic. The characteristics of this electrogenic process have been studied.

The ATP analogue *lin*-benzo-ATP, which has an extended adenosine ring was used to study substrate specificity of the phosphorylation reaction and the effect on the low affinity binding site of ATP. Phosphorylation of $(Na^+ + K^+)$-ATPase with this modified substrate was similar to that with ATP, but the extension of the adenosine ring appeared to have severe implications for the binding on the low affinity binding site and cation transport as tested with reconstituted $(Na^+ + K^+)$-ATPase. In contrast to $(Na^+ + K^+)$-ATPase, $(H^+ + K^+)$-ATPase was not able to hydrolyse this synthetic substrate, but the substrate for this enzyme appeared to be a tool to distinguish between the basal Mg^{2+}ATPase activity and the K^+-stimulated ATPase activity.

Although all "P"-type ATPases can be phosphorylated by inorganic phosphate (P_i) not much attention has been paid to this reaction for $(H^+ + K^+)$-ATPase in contrast to $(Na^+ + K^+)$-ATPase. The characteristics of this reaction have been studied with special attention on the role of K^+ and the K^+ antagonistic inhibitor of $(H^+ + K^+)$-ATPase, SCH 28080.

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Chapter 2

Reconstitution of $(Na^+ + K^+)$-ATPase

SUMMARY

Reconstitution procedures for membrane bound enzymes described in the literature were applied to reconstitute purified rabbit kidney $(Na^+ + K^+)$-ATPase in lipid vesicles. The results of several procedures were compared with respect to the effectiveness of the reconstitution of rabbit kidney $(Na^+ + K^+)$-ATPase. The freeze-thaw-sonication method in combination with liposomes which were prepared with the reverse phase evaporation method, gave the best results. This method was further elaborated and optimized for reconstitution of $(Na^+ + K^+)$-ATPase by variation of several parameters. The resulting proteoliposomes were characterized and appeared to have relatively high phosphorylation, hydrolytic and transport capacities.

INTRODUCTION

Important information has been derived from structural and mechanistic studies carried out with isolated membrane fragments containing $(Na^+ + K^+)$-ATPase These membrane fragments consist of a lipid bilayer and a protein part and contain more than 95% $(Na^+ + K^+)$-ATPase (1) The disadvantage of this enzyme preparation is the loss of sidedness. This makes it impossible to study the most important feature of the enzyme cation transport across the bilayer Another disadvantage is the impossibility to assign effects of ligands on $(Na^+ + K^+)$-ATPase to one side of the enzyme i e to determine the sidedness of the effects Transport and sidedness studies are possible in preparations with the enzyme present in a closed vesicle structure single cells e g erythrocytes, inverted red blood cells or the microsome fraction of the enzyme preparation The disadvantage shared by these preparations, however, is the lower extent of purification of the enzyme compared to the membrane fragments. The vesicles or cells contain many other membrane bound proteins, obscuring the assignments of properties and effects to the enzyme of interest, $(Na^+ + K^+)$-ATPase

Disadvantages of both systems can be overcome by reconstitution of the enzyme Reconstitution is here defined as the incorporation of a purified membrane bound enzyme, in this case $(Na^+ + K^+)$-ATPase, in a liposome of known lipid composition In the reconstituted system the sideness of the enzyme is regained and contaminating proteins are nearly absent

In the previous chapter an overview of possible procedures for reconstitution has been given This chapter describes some of these procedures to reconstitute $(Na^+ + K^+)$-ATPase isolated from rabbit kidney outer medulla A comparison of the methods with respect to this enzyme will be given and properties of the proteoliposomes will be discussed. This chapter will also give an empirical rationale for the reconstitution procedure used in this thesis

MATERIALS AND METHODS

Preparation of $(Na^+ + K^+)$-ATPase

$(Na^+ + K^+)$-ATPase from rabbit kidney outer medulla has been prepared according to the method described by Jørgensen (2) About 340 mg microsomes (on protein base) were incubated for one hour at 20° in a medium containing 0 58 mg/ml sodium dodecyl sulfate and 25 mM imidazole-HCl (pH 7 4), 3 mM ATP and 2 mM EDTA (final protein concentration 1 45 mg protein per ml) After the extraction the microsomal suspension was centrifuged on a sucrose gradient

(0-50%). The ATP of the pooled fractions of the gradient was removed by incubation at 37° in the presence of Na^+, Mg^{2+} and K^+ and subsequent washing. The obtained membrane fragments, enriched in (Na^++K^+)-ATPase, were stored in imidazole buffer (25 mM, pH 7.4) containing 10% sucrose (w/v). The specific (Na^++K^+)-ATPase activity of the preparations ranged from 1.0 to 1.6 mmol P_i formed mg^{-1} protein per hour.

Protein determination

The protein content of the purified enzyme was determined either by the method of Lowry et al (3) or with the use of a fluorimeter with an exitation and an emission wavelength of 278 and 340 nm respectively. Since the high lipid content of the proteoliposomes interfered with the Lowry protein determination, the protein content of the reconstituted (Na^++K^+)-ATPase was determined with the fluorescent method.

Lipid phosphorus measurement

Phosphate content of the liposomes was determined with the method of Fiske and Subba-Row (4). Samples of 100 µl were digested with 0.2 ml concentrated H_2SO_4/$HClO_4$ for one hour at 180°C. The tubes were then cooled below 50°C. In case the destruction was incomplete, 0.1 ml 30% H_2O_2 was added and the destruction continued until the samples were colourless. After cooling, 4.75 ml of a freshly prepared mixture of 50 ml of a solution containing 2.60 g $(NH_4)_6Mo_7O_{24}.4H_2O$ and 2.2 ml of a solution containing 30.1 mg $Na_2S_2O_5$ + 11 mg $Na_2S_2O_3$ + 55 mg aminonaphtalene sulfonic acid was added. The contents of each tube were mixed and incubated for 20 min in a boiling water bath. After cooling with tap water and standing for 30 min, the 820 nm absorbance was measured against water. In each determination a series of standard P_i samples was incubated and similarly treated.

Solubilization

Membrane preparations of (Na^++K^+)-ATPase were incubated with different concentrations of detergents. Thereafter the detergent was either removed by filtration on a Sephadex G-25 column (5) or diluted to ineffective concentrations.

Preparation of liposomes

Liposomes were prepared by a reversed phase evaporation method as described by Szoka and Papahadjopoulos (6). Mixtures of cholesterol, phosphatidylcholine and phosphatidylserine (ratio indicated in text and legends) in chloroform were evaporated under a stream of nitrogen to remove the organic solvent. After repeated washing with diethylether, a 1:1 mixture of diethylether and buffer solution of different composition was added and the solution thoroughly mixed on a Vortex mixer, while the diethylether was again slowly evaporated by a stream of nitrogen. The final lipid content was between 20 and 50 mg/ml. After all ether had disappeared, the liposomes formed were sonicated for 30 min in a Branson sonicator bath at maximal output.

Determination of the vesicle volume

With the use of radioactive tracer

Liposomes were prepared in the presence of $^{86}Rb^+$, resulting in a homogenous distribution of the tracer in the intra- and extravesicular space. The tracer in the extravesicular medium was removed by elution on a cation exchange resin (as described for the transport assay). The quantity of tracer in the void volume of the effluent was taken as a measure for the intravesicular volume. This could be calculated after subtraction of the blank (the radioactivity present in the effluent of the $^{86}Rb^+$ solution containing no liposomes).

Fluorometric assay

According to the method of Oku et al (7) 5 µl of a liposome suspension prepared in the presence of the fluorescent probe calcein (0.1 mM) was diluted with 1 ml buffer solution of equal osmolarity and the fluorescence was measured on a Shimadzu RP 510 spectrofluorimeter (exitation wave length 490, slit 10 nm; emission wavelength 520 nm, slit 10 nm) before (F_{tot}) and after (F_{in}) addition of 5 µl of a 10 mM $CoCl_2$ solution. Subsequently 25 µl of a 10% Triton X-100 solu-

tion in buffer was added and the fluorescence was measured again (F_{totq}). The trapped volume was calculated from $[F_{in} - (F_{totq} * r)]/[F_{tot} - (F_{totq} * r)] * 100 = \%$ trapped volume. In this formula the r stands for the dilution factor caused by addition of Triton X-100. Dilution due to addition of the $CoCl_2$ solution has been neglected.

Reconstitution procedures

Cholate-Dialysis

A lipid solution was prepared by drying 20 mg pure phosphatidylcholine under a stream of nitrogen and resuspending the lipid in one ml of a solution containing 30 mM imidazole/HCl (pH 7.4), 1 mM EDTA, 5 mM $MgSO_4$ and 0.8% (w/v) cholate. An equal volume of purified $(Na^+ + K^+)$-ATPase in the same buffer solution was added to the lipid suspension and mixed during 30 s. Thereafter the mixture was transferred to a dialysis tube and dialized during 90 h at 4°C against the above mentioned buffer without cholate.

Gel filtration

According to the method of Abeywardena et al (8) 50 mg a crude phosphatidylcholine solution in chloroform was dried under a stream of nitrogen and resuspended in 1 ml of a buffer solution containing 20 mM Tris/HCl (pH 7.4), 1mM EDTA, 5 mM $MgSO_4$ and 1% (w/v) deoxycholate. The suspension was mixed at room temperature until it became translucent. of a suspension of purified $(Na^+ + K^+)$-ATPase in the same buffer 0.2 ml was added to the lipid/detergent mixture. After mixing with a Vortex mixer for one min at room temperature, the enzyme/lipid/detergent mixture was chromatographed on a column of Sephadex G-25 in the above mentioned buffer. The void volume containing lipid and protein was used in the phosphorylation assay.

In a similar method (9) dried phosphatidylcholine and cholesterol (85:15 w/w) were resuspended in a buffer solution containing 25 mM histidine (pH 7.0), 1 mM EDTA, 5mM Mg^{2+} and 1 mM dithioerytreitol. After sonication 5% (w/v) Chaps was added to the lipid suspension (final lipid concentration 50 mg/ml). The mixture was incubated for 15 min at 15°C and subsequently diluted twice in the buffer solution. Purified $(Na^+ + K^+)$-ATPase (1.2 mg/ml) was solubilized at 15°C for 5 min in the presence of 5.5% (w/v) Chaps in the same buffer solution. Thereafter 14 volumes of the lipid/detergent were added to the solubilized protein. After incubation at 15°C for 5 min the detergent was removed from this mixture by gel filtration (Sephadex G-25) as described by Penefsky (5).

Freeze-Thaw

Based on the procedure of Karlish and Pick (10) liposomes were prepared by sonication of a mixture of phosphatidylcholine and cholesterol (85:15 w/w) in a buffer solution containing 25 mM imidazole/HCl (pH 7.4), 1 mM EDTA and 5 mM Mg^{2+}. 45 µl Of the enzyme suspension (5 mg/ml) was solubilized on ice in the presence of 0.8% (w/v) cholate during one min. To the solubilized enzyme 450 µl liposomes (lipid content 34 mg/ml) was added. After mixing this lipid/protein/detergent mixture was rapidly frozen in liquid nitrogen and allowed to thaw slowly at room temperature. Finally the detergent was removed by gel filtration on a Sephadex G-25 column (5).

Freeze-Thaw-Sonication

Purified $(Na^+ + K^+)$-ATPase (5 mg/ml) in 20 mM imidazole/HCl buffer (pH 7.2) was partially solubilized by incubation with cholate (final concentration 0.91% (w/v)) during 1 min at room temperature. This mixture was then added to a ten-fold volume of liposomes. After thorough mixing, the preparation was frozen in liquid nitrogen or in a mixture of dry ice and acetone and subsequently thawed at room temperature. Thereafter the vesicle suspension was sonicated for six minutes in a Branson sonicator bath (maximal output). Detergent was removed from the proteoliposomes by centrifuging aliquots of the suspension over a 10-fold volume Sephadex G-25 (coarse) column (equilibrated with the appropriate buffer solution) in a syringe.

Gradient centrifugation

The reconstitution mixture (before the gel filtration step) was layered on top of a discontinuous gradient of 0, 10, 20 and 40% sucrose (w/v) as described before (11). The gradient was centrifuged for 90 min at 80,000 g in a TST 28-38 swing out rotor (MSE centrifuge). Afterwards fractions of 1.5 ml were collected from the top of the tube and the pattern at 280 nm was measured with an Uvicord UV cell.

ATP hydrolysis

Coulorimetric method

Na^+, K^+ and Mg^{2+} stimulated ouabain sensitive ATPase activity was determined as the difference in ATP hydrolysis in two media A and E (12). The total ATPase activity and the ouabain insensitive ATPase activity were measured in media A and E, respectively. Medium A contains 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP and 50 mM imidazole/HCl (pH 7.4). Medium E has the same composition and pH as medium A except that KCl was omitted and 0.1 mM ouabain was added. 10 µl Enzyme suspension was incubated in 0.4 ml of one of the media at 37°C. The reaction was stopped by addition of 1.5 ml cold 8.6% (w/v) trichloroacetic acid. Then 1.5 ml of a freshly prepared solution of 9.6% (w/v) $FeSO_4.6H_2O$, 1.15% (w/v) ammonium heptamolybdate in 0.66 M H_2SO_4 was added. After 30 min standing at room temperature the activity was calculated from the extinctions of the probes and standard inorganic phosphate solutions at 700 nm. For blanks values 1.5 ml trichloroacetic acid was added to medium E prior to the enzyme suspension.

Radioactive method

The Na^+ and K^+ stimulated ATP hydrolysis was determined as the release of $^{32}P_i$ from [$\gamma^{32}P$] ATP (33). To 10 µl proteoliposomes containing Na^+, Mg^{2+} and K^+, in Tris buffer (pH 7.2), 190 µl of a medium containing Na^+, Mg^{2+}, K^+, Tris (pH 7.2), ouabain (0.2 mM) and 0.5 and 1.0 mM labeled ATP were added at room temperature. The Na^+ stimulated ATP hydrolysis of proteoliposomes without intravesicular K^+ was determined in a similar way. The extravesicular medium contained 1 (or 5) µM labeled ATP and was free of K^+. For blank values the reconstituted (Na^++K^+)-ATPase was denatured with trichloroacetic acid prior to incubation with the assay medium. The $^{32}P_i$ production was measured after stopping the reaction at a given time by addition of 0.4 ml 10% trichloroacetic acid followed by mixing with 0.4 ml 20% (w/v) aqueous charcoal suspension. The charcoal adsorbs the adenosine phosphates from the medium, but leaves P_i in solution. The suspension was mixed thoroughly during 10 s each 5 min (3 times). Thereafter the charcoal was sedimented by centrifugation for 10 min at 2000 g at 0°. Aliquots (0.2-0.5 ml) were taken from the supernatant, mixed with 4.5 ml liquid scintillation fluid (Aqualuma Plus). Radioactivity was measured with a liquid scintillation counter.

Transport assays

Rb^+ transport

For transport studies proteoliposomes loaded with 20 mM K^+, were equilibrated with a medium containing $^{86}Rb^+$ (0.1 mM) during 3 hours at room temperature (10). After loading with the tracer the proteoliposome suspension was incubated with a 10-fold volume of transport medium containing Na^+, K^+, Mg^{2+} and ouabain, with or without ATP. After the incubation at room temperature the transport was quenched by layering an aliquot of the suspension on a Dowex-50 x8 (Tris-form) column (13). The proteoliposomes were then eluted with 1 ml of a solution of 250 mM sucrose and 25 mg/ml bovine serum albumin. The eluate, containing proteoliposomes devoid of the external Rb^+, was counted in a liquid scintillation counter by measuring Cerenkov radiation.

Fluorometric assay

Fluorescence of a membrane potential sensitive probe Oxonol VI was measured in a thermostrated cuvette holder equipped with a magnetic stirrer on a Shimadzu 510 fluorescence spectrophotometer, at exitation and emission wavelengths of 580 and 660 nm respectively (15). The fluorescence of 150 nM Oxonol VI in presence of proteoliposomes was taken as F_O and the change

TABLE I

Method	detergent	% total phosphorylation level	% ouabain insensitive phosphorylation level
dialyis	cholate	5	4 (80)
gel filtration	cholate	12	11 (92)
gel filtration	deoxycholate	30	20 (67)
gel filtration	deoxycholate (+cholesterol)	38	29 (76)
gel filtration	Chaps	19	17 (89)
freeze-thaw	deoxycholate	37	27 (73)
freeze-thaw		40	28 (70)
freeze-thaw-dilution	cholate	42	4 (10)
freeze-thaw-sonication	cholate	50	35 (70)

Table I

Recovery of the phosphorylation level of reconstituted (Na^++K^+)-ATPase in the presence (third column) and the absence (fourth column) of ouabain as percentage of the phosphorylation level of the untreated (Na^++K^+)-ATPase in the absence of ouabain. In brackets (fifth column) the percentage of phosphorylation level of reconstituted (Na^++K^+)-ATPase which remains after preincubation with 0.2 mM ouabain is given (The ratio of the figures in column four and three x 100%) For details see Materials and Methods and the text

in fluorescence after addition of ATP to the medium F_t $F_o/F_t = \Delta F$ was recorded as the time dependent change in membrane potential due to active pumping of reconstituted $(Na^+ + K^+)$-ATPase

Phosphorylation

Phosphorylation of the reconstituted $(Na^+ + K^+)$-ATPase was carried out at 22° at pH 7 0 The ATP concentrations varied between 0 2 and 20 µM (The Radiochemical Centre, Amersham, UK , specific radioactivity 3000 Ci/mole) The reaction was started by rapid mixing of 10 µl proteoliposomes (preincubated with 0 2 mM ouabain and 10 mM Mg^{2+}) with 90 µl of the medium containing ATP and the other ligands The reaction was stopped after 3 s by addition of 3 ml 5% (w/v) trichloroacetic acid, containing 100 mM phosphoric acid The denatured phosphoprotein was filtered on a 1 2 µm pore width Selectron filter (Schleicher and Schull, Dassel FRG), which was then washed three times with 3 ml of the stopping solution Incorporated ^{32}P was determined by liquid scintillation counting For blank values the proteoliposomes were mixed with the stopping solution prior to addition of the ATP solution

Since many parameters play a role in the whole procedure inter-experimental reproducability was much lower than with fragmented enzyme Deviations of less than 50% between independently prepared proteoliposomes have been tolerated When different proteoliposomes batches were prepared in parallel the deviations were reduced to less than 10% Reproducebility in experiments with one proteoliposome batch was similar to experiments with fragmented enzyme

Materials

ATP and Tris were purchased from Boehringer, Mannheim, F R G $[\gamma^{32}P]$ATP and ^{86}Rb were obtained from Amersham, Buckinghamshire, U K , phosphatidylcholine (egg) and phosphatidylserine (bovine brain) were purchased from Avanti Polar Lipids, Birmingham, AL, U S A , and cholesterol, from Sigma, St Louis, MO, U S A All other chemicals were of reagent grade.

RESULTS

Reconstitution procedures

Some of the reconstitution procedures described in the literature were applied for the reconstitution of the $(Na^+ + K^+)$-ATPase preparation from rabbit kidney outer medulla In all of the methods described here the following steps were used

i Solubilization of the enzyme
ii Mixing lipids or preformed lipid vesicles with the enzyme
iii Manipulations like freezing and thawing and sonication
iv Removal of the detergent

For comparison the proteoliposomes obtained by the following procedures were tested for their phosphorylation capacity in the presence and absence of ouabain This gives an impression of the recovery of the enzyme during the reconstitution procedure and the amount of pump molecules incorporated in the inside-out orientation in tight lipid vesicles The phosphorylation levels obtained in our hands with the different procedures are described below and summarized in table I

Cholate dialysis

This procedure based on that of Anner and Moosmayer (16) yielded a low phosphorylation level in the absence of ouabain The ouabain insensitive part, however, was low Since the presence of ouabain does not affect inside-out incorporated enzyme, this indicates that most of the pump molecules were incorporated in the liposomes, but that a severe loss of enzyme activity had occurred

Gel filtration

Dependent on the detergent used a relatively low and high recovery of the enzyme activity was obtained by the method based on that of Abeywardena et al (8) 12 and 30% with cholate and

Addition, manipulation	optimum	remarks	tested with respect to:
cholate	0.05-0.3 mg/ml (protein 0.45 mg/ml)		Rb^+ transport
lipids	22-45 mg/ml (protein 0.45 mg/ml)		Rb^+ transport
freezing and thawing	3 times	relative activity after 1x 43% 2x 93% 3x 100% 4x 72%	hydrolysis
sonication	5 min	longer time inactivated the enzyme	hydrolysis
gel filtration	1 x	repetition decreased the activity due to loss of protein	hydrolysis

Table II

Effects of manipulations and additions during the reconstitution procedure of $(Na^+ + K^+)$-ATPase with the freeze-thaw-sonication method. Details are mentioned in the text.

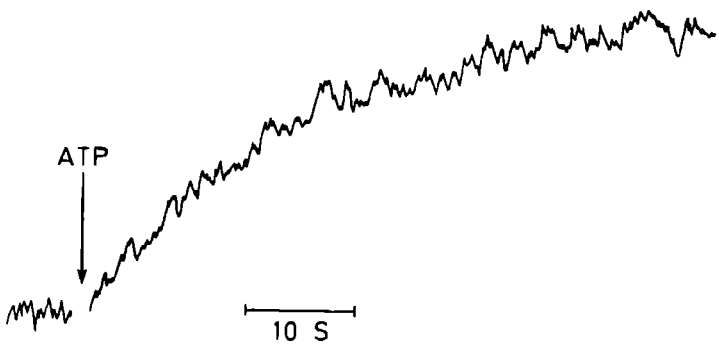

Figure 1 Membrane potential generated by reconstituted $(Na^+ + K^+)$-ATPase

Proteoliposomes containing intravesicularly 145 mM Na^+, 5mM K^+, 3 mM Mg^{2+}, 0 1 mM EDTA and 30 mM imidazole (pH 7 2) were incubated at 22°C in two ml of the intravesicular medium containing 150 nM Oxonol VI The arrow indicates the addition of 0 25 mM ATP The trace represents the fluorescence increase as a result of the membrane potential generated by the Na^+/K^+-pump The fluorescence is measured as described in the Materials and Methods section

deoxycholate respectively. Replacement of 15% of the phosphatidylcholine by cholesterol (on weight basis) resulted in a further increase of the phosphorylation level up to 38% of the untreated enzyme. With cholate more than 90% of this level was ouabain insensitive, whereas, with deoxycholate only 67% was ouabain insensitive. Addition of cholesterol increased the latter percentage to 76%.

With the method of Yoda et al (9), using Chaps, a phosphorylation level of 19% of that of the untreated enzyme was obtained. The major part of this level was not decreased by ouabain, indicating that the $(Na^+ + K^+)$-ATPase was indeed incorporated in the lipid vesicles.

Freeze-thaw

This method as described by Karlish and Pick (10), yielded a relatively high phosphorylation level of the reconstituted enzyme in our hands. In the absence of ouabain 37 and 40% of the phosphorylation level of untreated enzyme was obtained with deoxycholate and cholate as the respective detergents. In the presence of ouabain the levels were reduced to 27 and 28% respectively.

Freeze-thaw and dilution

When the detergent was not removed by the gel filtration step but simply diluted in the assay medium a somewhat higher phosphorylation level was obtained in the absence of ouabain (42 vs. 40%). In the presence of ouabain however less than 10% of the level remained, indicating that the presence of the detergent in the proteoliposomes made them leaky.

Freeze-thaw-sonication

Phosphorylation levels of up to 50% of the $(Na^+ + K^+)$-ATPase preparation before reconstitution have been obtained with this method (17). More than 75% of this level appeared to be ouabain insensitive.

Since the last procedure yielded the best results with respect to the recovery of the phosphorylation level it has been further elaborated. Attemps have been made to optimalize the procedure also with respect to hydrolytic activity of the enzyme and to transport capacity of the proteoliposomes.

Transport capacity of reconstituted $(Na^+ + K^+)$-ATPase

Cation transport of the proteoliposomes was studied by the measurement of ATP dependent $^{86}Rb^+$ fluxes out of the vesicles (as described in Materials and Methods). Although the time resolution of this method is bad, the rate of the Rb^+ transport was about two times as fast as the rate of ATP hydrolysis (hydrolysis rate 65 μmoles $P_i mg^{-1} h^{-1}$; Rb^+ transport rate ~100 μmoles $mg^{-1} h^{-1}$). This is in accordance with the concept of 2 K^+ ions translocated per hydrolyzed ATP molecule. With respect to initial rates of Rb^+ transport the freeze-thaw-sonication procedure has been optimized for the lipid:protein and the detergent:protein ratio. Highest rates have been obtained with 0.5-3 mg/ml cholate in the reconstitution mixture (protein content 0.45 mg/ml). The optimal protein:lipid ratio turned out to be between 1:50 and 1:100.

Since the $^{86}Rb^+$ transport assay has a bad time resolution it was desirable to measure active transport rates more accurately with another method. With the membrane potential sensitive fluorescent probe Oxonol VI the change in membrane potential can be followed in time. This (negatively charged) hydrophobic probe is accumulated in the vesicle when the membrane potential becomes positive (15). The increase in fluorescence indicated that a net flux of positive charge into the vesicle resulted from the pump activity of $(Na^+ + K^+)$-ATPase upon addition of ATP to the medium. With this probe it has been shown that the efflux of K^+ from the proteoliposomes was compensated by the net influx of possitive charge into the vesicle. The linearity of the increase of the fluoresence (Fig. 1) was less than 20 s whereas the rate of hydrolysis was linear for about 60 s. Since the method in this study has only been used in a qualitative way, it does not give information on stoichiometry of the cation transport by the reconstituted $(Na^+ + K^+)$-ATPase.

Effects of reconstitution steps in the freeze-thaw-sonication procedure on reconstituted and fragmented enzyme

The effect of detergents, lipids, freezing and thawing, sonication and gel filtration on the purified and reconstituted enzyme were studied and are qualitatively described here (Table II)

The first step in the reconstitution procedures is the detergent treatment of the enzyme in order to facilitate the incorporation into the bilayer of the liposome Therefore the effects of some detergents on $(Na^+ + K^+)$-ATPase have been studied and compared Incubation of the enzyme suspension (5 mg/ml) with the surfactant for 5 min on ice resulted in concentration dependent inactivation of the enzyme with all the detergents tested. After incubation the enzyme detergent mixture was diluted one hundred fold and the hydrolytic activity was measured with the coulorimetric assay The inactivating effects of cholate, deoxycholate, Chaps and $C_{12}E_8$ were half maximal at 0 9, 1 0, 0 8 and 0 08% (w/v) respectively

To test the extent of solubilization of the enzyme with low concentrations of detergent, enzyme preincubated with 0 9% cholate was centrifuged on discontinuous sucrose gradient in order to separate solubilized from non-solubilized enzyme Only 37% of the protein appeared in the upper band of the gradient and contained 13% of the activity of the untreated enzyme, whereas 63% of the protein was sedimented in the heavier band and contained 41% of the activity of the untreated enzyme Although the total amount of the enzyme did not change during the solubilization procedure, only 54% of the total activity was recovered

Repeated freezing and thawing of the lipid protein detergent mixture increased the amount of incorporated enzyme into the liposomes with an optimum of three times More freezing and thawing steps resulted in loss of activity of the reconstituted enzyme Freezing and thawing of the fragmented enzyme did not influence the hydrolytic activity of it

Sonication of the reconstitution mixture increased the activity of the proteoliposomes in the presence of ouabain with an optimum of 5 min Longer sonication resulted in loss of activity Sonication of a suspension of purified enzyme resulted in loss of activity after two min

Gel filtration on a Sephadex G 25 column resulted in formation of tight vesicles which in presence of ouabain showed higher activity than those of which the detergent concentration was reduced by dilution The detergent probably caused leakiness of the bilayer Repeated gel filtration caused loss of activity mainly by reduction of the protein content of the suspension Gel filtration of purified enzyme also resulted in loss of activity which was mainly due to loss of protein (up to 30% loss per filtration step) With proteoliposomes the loss in protein content was lower 20% in the first filtration step and about 5-10% in subsequent steps.

Addition of a suspension of phosphatidylcholine to purified $(Na^+ + K^+)$-ATPase reduced the activity of the enzyme significantly This inactivation could be overcome by addition of small amounts of bovine serum albumin, suggesting that the inhibition was caused by free fatty acids (18, 19) The presence of bovine serum albumin during the reconstitution procedure significantly increased the activity of the reconstituted $(Na^+ + K^+)$-ATPase

Characterisation of the liposomes

In the freeze-thaw-sonication procedure liposomes prepared by the reverse phase evaporation method have been used to incorporate the enzyme One parameter for the quality of the liposomes is the tightness To get an impression of the tightness of the vesicles the internal volume can be determined This has been done by three independent methods

In the first method the vesicles have been prepared in a medium containing $^{86}Rb^+$ After removal of the external medium by elution on a cation exhange resin the intravesicular volume could be calculated from the radioactivity present in the void volume of the eluent With this method a volume of 190 µl (representing 19% of the total volume) was calculated to be enclosed by the lipid vesicles (50 mg lipid per ml)

In a second approach liposomes have been prepared in the presence of the fluorescent probe

calcein (7). Based on the assumption that the calcein concentration was the same inside and outside the vesicles, the internal volume could be determined by quenching the calcein fluorescence of the external medium with Co^{2+}. A vesicle volume of 18-22% (of the total volume) was determined by this method (see Materials and Methods).

Finally the vesicle volume was derived from the diameter of the freeze fractured liposomes on E.M. photographs. From this diameter the volume of 18% has been derived with the assumption of a membrane thickness of 5 Å.

From the volume, lipid content, lipid density and membrane thickness the number of vesicles could be estimated. From this figure together with a molecular weight of 150 kDa of the $\alpha\beta$ protomer the number of monomers per proteoliposome can be calculated. With 5 mg/ml protein and 50 mg/ml lipids an average of one to two $\alpha\beta$ units per vesicle was derived.

With the fluorescent method effects of several manipulations during the reconstitution procedure on the vesicle integrity have been tested. Repeatedly freezing and thawing in first instance increased the vesicle volume slightly, but did not change it after the second time. Prolonged sonication decreased the vesicle volume after 30 min. Disturbing the integrity of the vesicles was possible by addition of detergents. Several surfactants have been tested for their effectiveness of opening the vesicle. Cholate, Chaps and sodium dodecyl sulphate opened the vesicles with half maximal concentrations of 0.1, 0.1 and 0.16% (w/v), repectively. Opening of the vesicles was also carried out with the $^{86}Rb^{+}$ loaded vesicles. Cholate opened the vesicles with a total loss of internal $^{86}Rb^{+}$ with a half maximal effect at 0.13% cholate (w/v). From these results it is clear that the vesicle integrity has been destroyed and that Co^{2+} and calcein (and probably also ATP, and cations and ouabain) can perturb the bilayer, but it is impossible to conclude whether the vesicles are made leaky or permeable for certain compounds or whether the vesicle structure has been destroyed totally.

Characterisation of the proteoliposomes

Na^{+}-stimulated hydrolytic activity

Proteoliposomes loaded with Na^{+} but free of K^{+} were used for the determination of the rate of hydrolysis of the Na^{+}-stimulated ATPase activity (20). The rate of the Na^{+}-stimulated ATP hydrolysis was 1.6 µmol P_i per mg per h at 20°C. This represents 13% of the activity of the untreated (Na^{+}+K^{+})-ATPase under the same conditions.

Na^{+}- + K^{+}-stimulated hydrolytic activity

Proteoliposomes loaded with Na^{+} and K^{+} ions can exchange intravesicular K^{+} for extravesicular Na^{+} when Na^{+}, Mg^{2+} and ATP are present in the extravesicular medium. The pump activity increased with a factor of 25 compared to the K^{+} free proteoliposomes. The hydrolytic activity became 65 µmol P_i per mg protein per h., which was 20% of the hydrolysis of the untreated (Na^{+}+K^{+})-ATPase under the same enzyme conditions. The linearity of the hydrolysis with respect to to time lasted not longer than 60 s. Addition of ionophores did not significantly change the initial velocities, but some of them increased the linearity of the P_i production in the time. Monensin was without effect and valinomycin only slightly increased linearity, whereas nigericin and a combination of valinomycin and FCCP prolonged the linearity to 2 min.

Incorporation and orientation of the reconstituted (Na^{+}+K^{+})-ATPase

Although it is desirable to have as many pump molecules as possible with their ATP binding site oriented to the extravesicular side, no special care has been taken to influence the orientation of the pump molecules in the proteoliposomes. The advantage of the inside-out orientation (i.e. the opposite orientatation as in the in vivo situation which is denominated rightside-out) is that the substrate added to the medium can reach the reconstituted enzyme. After reconstitution a part of pump molecules is not incorporated in a closed bilayer. This part of the population is

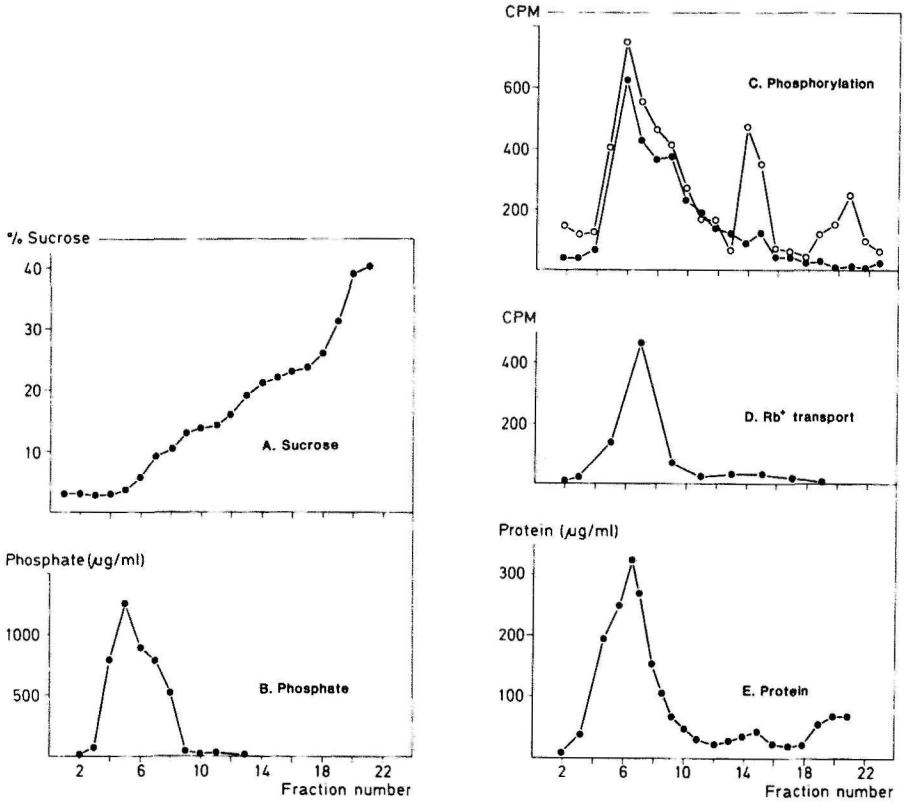

Figure 2. Separation of reconstituted (Na^++K^+)-ATPase on a discontinuous sucrose gradient

Panel A shows the sucrose density of 1.5 ml fractions after 90 min centrifugation. The phosphate content (panel B) was determined after heat destruction of the fractions as described in Materials and Methods. The relative phosphorylation level (panel C) was determined in the absence (o) and the presence (•) of ouabain. In panel D the active extrusion of Rb^+ initiated by ATP during the first 30 s of incubation at 22°C is represented. The protein content of the fractions was determined fluorometrically (panel E). For further details see text and the Materials and Methods section.

called not-incorporated and is sensitive to inhibition by ouabain. The inside-out oriented population is insensitive to ouabain in the medium since they have their ouabain binding site located in the vesicle interior. The rightside-out oriented pump molecules have their ATP binding site inside the vesicle and can not bind ATP in the medium and are therefore silent in most assays. In the absence of ouabain inside-out and not-incorporated enzyme molecules can be phosphorylated by ATP. In the presence of ATP not-incorporated enzyme molecules are blocked by the inhibitor and only inside-out oriented (Na^++K^+)-ATPase can be phosphorylated. By opening the vesicles with a detergent ATP can penetrate the proteoliposomes and also the rightside-out oriented pump molecules can be phosphorylated. With ouabain present intravesicularly the inside-out oriented pump-molecules are blocked with respect to phosphorylation and only not-incorporated (Na^++K^+)-ATPase can be phosphorylated. The opening of the vesicles with a detergent has the disadvantage of inactivation of the enzyme. With cholate, however, a narrow concentration range (0.2-0.5%, w/v) has been found where vesicles are opened and inactivation is minimal. In the absence of ouabain detergent treated and untreated proteoliposomes have been phosphorylated in order to obtain the ratio of inside-out+not-incorporated:total. Untreated proteoliposomes have been phosphorylated in the presence and absence of ouabain in order to find the ratio of inside-out:inside-out+not-incorporated. From these two ratios a ratio of 38 ± 16 : 36 ± 14 : 26 ± 12 (mean ± S.D., n=5) for inside-out : rightside-out : not-incorporated has been obtained.

Another way to determine the ratio of inside-out incorporated and not-incorporated (not-incorporated) enzyme molecules is to separate the proteoliposomes from the free enzyme fragments. To obtain this separation 1 ml of the reconstitution mixture was centrifuged on a discontinuous sucrose gradient before the removal of the detergent by gel filtration. After 90 min centrifugation three bands at densities of 5-10, 21-23 and more than 40% sucrose had formed. Although after 90 min equilibrium was not yet reached, longer centrifugation caused diffusion of the bands. All fractions of the gradient were collected and tested for their phosphorylation capacity in the presence and absence of ouabain, and for their transport capacity. Furthermore the protein and lipid contents of the fractions were determined. A typical pattern of the gradient is shown in fig. 2. The lightest band contained practically all lipids and protein and the two heavier bands contained mainly protein with a (not detectable) amount of lipids. All bands contained enzyme activity in the absence of ouabain. The presence of all lipids indicated that the first band contained proteoliposomes and the second and third band not-incorporated (Na^++K^+)-ATPase. Judged by the densities the second and third band contained solubilized and not-solubilized enzyme, respectively. That the first band contained proteoliposomes was further confirmed by the enzymatic parameters. The phosphorylation and hydrolytic activity of the heaviest band were insensitive to ouabain, whereas the second and third bands were inhibitable by ouabain. Moreover the transport capacity was concentrated exclusively in the lightest band. The protein content of the heaviest and middle band was about 15% of the total protein.

DISCUSSION

Comparison of several procedures for the reconstitution of (Na^++K^+)-ATPase used in this study yields a result which is in accordance with the general view in the literature: cholate-dialysis yielded good vesicles with however, a low recovery of enzyme activity. The decrease in activity is probably due to the long exposure of the enzyme to the detergent. In other procedures in which the incubation time of the enzyme with the detergent was much shorter the loss of enzyme activity was considerably less. With gel filtration methods in which proteoliposomes were formed during rapid removal of detergent by a chromatographic step much higher activities of the reconstituted enzyme were obtained. Additionaly freezing and thawing and sonication of the

reconstitution mixture further improved the reconstitution The method of Cornelius and Skou (20) has not been applied, since the detergent used by these authors, $C_{12}E_8$, caused in our hands irreversible inactivation of the rabbit kidney $(Na^+ + K^+)$-ATPase far below the concentration necessary for reconstitution as described for the $(Na^+ + K^+)$-ATPase from rectal glands of spiny dogfish (20) The dilution method of Racker et al (21) which has been applied in combination with the freeze-thaw procedure (dilution of the detergent instead of removal by gel filtration) yielded high phosphorylation levels in the absence of ouabain Only a small fraction (less than 10%) of the enzyme, however, was insensitive to ouabain, indicating that only a small part of the pump molecules was incorporated in the vesicles or that the vesicles were leaky in the presence of low concentrations of detergent remaining in the vesicles after dilution

The method which gave the best recovery of ouabain insensitive phosphorylation in our hands, the freeze-thaw-sonication procedure, has been optimized further Since a similar method was already used for the reconstitution of $(H^+ + K^+)$-ATPase (17) it was not necessary to work out the whole procedure again Therefore the method has been adapted for reconstitution of $(Na^+ + K^+)$-ATPase from rabbit kidney and the different steps in the procedure on the enzyme activity of $(Na^+ + K^+)$-ATPase have been controlled The most reproducable results were obtained with a 1 1 8 100 ratio of protein detergent lipid in the reconstitution mixture

Some operations of the freeze-thaw-sonication procedure influenced the activity of purified $(Na^+ + K^+)$-ATPase Prolonged sonication, gel filtration and incubation with the detergent reduced the activity of the fragmented enzyme, whereas freezing and thawing did not influence enzyme activity With the cholate concentration used (0 91%), only partial solubilization of the enzyme was obtained (37%) This concentration appeared, however, to be sufficient to facilitate incorporation of the enzyme into the bilayer (22) Higher detergent concentrations resulted in lower recovery of enzyme activity, probably due to the inactivating effect of the detergent

The freeze-thaw-sonication procedure using the lipid composition as described above, in which the freezing and thawing step was repeated three times and with 5 min sonication resulted in reconstitution of $(Na^+ + K^+)$-ATPase with incorporation of 75-90% of the enzyme in the lipid vesicles, a random orientation and a recovery of the activity of the inside-out oriented pump molecules of 60-80% The amount of not-incorporated enzyme determined with the ouabain/cholate method was higher than with the gradient centrifugation (25% versus 15%) Possibly the amount of not-incorporated has been overestimated with the first method due to ouabain leaking into the proteoliposomes which inhibits also inside-out oriented $(Na^+ + K^+)$-ATPase molecules Alternatively the not-incorporated population has been underestimated with the centrifugation method A possible reason for this underestimation could be that the not-incorporated $(Na^+ + K^+)$-ATPase molecules are partially centrifugated along with the proteoliposomes because they stick to the bilayer

The recovery of the phosphorylation levels was 30%, whereas that of the hydrolytic activity was maximaly 20% The overall reaction is probably more inactivated by the reconstitution procedure than the phosphorylation reaction Alternatively the lower turnover number of the reconstituted enzyme is due to the new lipid environment in the proteoliposome (see chapter 3)

Since reconstituted $(Na^+ + K^+)$-ATPase transported Rb^+ actively out of the proteoliposomes it must be concluded that the pump molecule is incorporated in the membrane in a proper way i e the $(Na^+ + K^+)$-ATPase spans the bilayer The transport of Na^+ has not been studied The results of the fluorescence transport experiment (together with the fact that Rb^+ and therefore K^+ can be transported by the reconstituted $(Na^+ + K^+)$-ATPase), however indicated that more Na^+ is transported in the inward direction than K^+ in the outward direction since a positive potential was created due to the pump activity of the inside-out oriented pump molecules If less or no Na^+ were transported against K^+ a negative potential would have been created

Since this assay is used as a qualitative one, nothing can be said about the stoichiometry From the fluorescence transport experiments it can be seen that the final membrane potential upon addition of ATP is reached within seconds, whereas the hydrolysis reaction is linear for about 30 seconds This could mean that the electric potential is not the only parameter responsible for inhibition of the activity of of the reconstituted (Na^++K^+)-ATPase and that after a longer time the chemical potential together with the electric potential are inhibitory.

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Lipid dependency of $(Na^+ + K^+)$-ATPase reconstituted in artificial lipid vesicles

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Submitted for publication Biochim. Biophys. Acta

footnote:

*The term "concentration" has been used throughout this paper to express the relative lipid content in the (proteo)liposome suspension.

Abbreviations:

Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenedinitrotetraacetic acid.

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SUMMARY

The lipid dependency of rabbit kidney $(Na^+ + K^+)$-ATPase reconstituted in proteoliposomes has been determined. The phosphorylation level in the presence of ouabain of the proteoliposomes with different lipid composition has been taken as a figure for the number of inside-out oriented $(Na^+ + K^+)$-ATPase molecules. From the hydrolytic activities in the presence and the absence of K^+ of the reconstituted $(Na^+ + K^+)$-ATPase (in the presence of ouabain) together with the phosphorylation level the molar activity of the inside out oriented $(Na^+ + K^+)$-ATPase molecules has been determined. Active $(Na^+ + K^+)$-ATPase was obtained after reconstitution with liposomes containing phosphatidylcholine as the only lipid compound. Partial replacement of phosphatidylcholine by cholesterol or by the neutral phospholipid phosphatidylethanolamine did not change the molar activity significantly. The negatively charged phospholipids phosphatidylserine, phosphatidylinositol and phosphatidic acid enhanced the molar activity when they were present in the proteoliposomes. These findings indicate that phosphatidylethanolamine and cholesterol have no influence on the molar activity compared to phosphatidylcholine and that negatively charged phospholipids stimulate the activity of $(Na^+ + K^+)$-ATPase but are not absolutely required for the activity of the enzyme.

INTRODUCTION

The membrane bound cation pump $(Na^+ + K^+)$-ATPase needs a hydrophobic environment for its activity. Delipidation of the enzyme leads to its inactivation (1-3). Relipidation of the enzyme with phospholipids or other amphipathic compounds like alkyl phosphate (4) restores the activity of the ATPase partially or totally (5, 6). The question whether there is a need for specific lipids has often been raised in the literature, but an unequivocal answer has not emerged yet (for a review of the older literature see ref 7).

Some authors state that specific phospholipids like phosphatidylserine (3, 8-12), phosphatidylinositol (11, 13, 14) or phosphatidylglycerol (9) are necessary for the activity of the enzyme. Tsakiris and Delinconstantinos (15) observed a stimulation of the $(Na^+ + K^+)$-ATPase activity in synaptosomal plasma membranes by phosphatidylserine. Other authors reported that just the negative charge of the phospholipid is important (1-3, 16-18). That the negative charge is important for reactivation is indicated by the failure of reactivation by phosphatidylcholine alone compared to the clear reactivation by phosphatidylcholine in

the presence of a negative charge provided by e.g. dicetyl phosphate or oleic acid (19) or negatively charged detergents (13).

These observations contrast to those of De Pont et al (20, 21) who stated that the presence of negatively charged phospholipids is of minor importance for the activity of the ATPase. In accordance with the latter authors is the occurrence of coupled Na^+ and K^+ transport in $(Na^+{+}K^+)$-ATPase reconstituted in lipid vesicles which contain phosphatidylcholine as the only lipid (22). Also Ottolenghi (23) reported that phosphatidylcholine is the best reactivator of $(Na^+{+}K^+)$-ATPase after delipidation. Cornelius and Skou (24), however, needed negatively charged phospholipids in their reconstituted system for full activity. It has also been suggested that phosphatidylcholine and phosphatidylserine are important activators of the $\alpha(+)$ isoenzyme, but have much less influence on the α form (25). An inhibitory effect of phosphatidylinositol in synaptosomal $(Na^+{+}K^+)$-ATPase has been reported by Nishikawa et al (26).

For cholesterol, another important lipid, there is neither a clear view on its role in the activity of $(Na^+{+}K^+)$-ATPase. Cholesterol was considered to be essential since after (partial) delipidation (27, 28) or oxidation of cholesterol (29) reactivation was achieved by the addition of this lipid compound. Stimulation of the $(Na^+{+}K^+)$-ATPase activity in erythrocyte ghosts by low cholesterol concentrations and inhibition by higher concentrations were observed by Yeagle (30). In contrast to the above findings an inhibitory effect by cholesterol on the activity of $(Na^+{+}K^+)$-ATPase has also been reported (12, 31). It was supposed that the cause for inhibition was the reduction of the membrane fluidity by cholesterol. Removal of cholesterol in erythrocyte membranes led indeed to an increase in the activity of $(Na^+{+}K^+)$-ATPase. It was also reported (32) that a decrease of the membrane fluidity by amiodarone inhibited $(Na^+{+}K^+)$-ATPase in rat brain synaptic membranes. The failure of some agents which increase the membrane fluidity to induce an increase of the enzyme activity argued, however, against membrane fluidity as the only factor in the mechanism of inhibition of $(Na^+{+}K^+)$-ATPase activity by cholesterol (33). Other authors, however, challenged a possible role of cholesterol and stated that it is non-essential for the function of $(Na^+{+}K^+)$-ATPase since the removal (34) or enzymatic modification did not change the enzymatic activity of the enzyme significantly (35).

One of the approaches used to study the lipid dependency of the enzyme is relipidation after delipidation of the protein. A problem with this method is that the extent of relipidation and the nature of the interaction between the lipid and protein is unknown. In an alternative approach the lipids are removed or modified either chemically or enzymatically. The problem with these procedures lies in the unknown nature of the side effects induced by the enzymes or chemical agents. When, however the enzyme is functionally incorporated in the lipid bilayer (i.e. when the protein spans the bilayer) the lipid must surround the protein in a fashion similar to the natural situation. In a preparation of $(Na^+{+}K^+)$-ATPase, reconstituted in lipid vesicles forming a proteoliposome, which is able to transport cations such a situation is present (36). The effects of the phospholipid composition and the role of cholesterol on the phosphorylation level and the turnover of the inside-out incorporated enzyme have been studied in such preparation. Phosphatidylethanolamine has been taken as an example for a neutral phospholipid and phosphatidylserine, phosphatidic acid and phosphatidylinositol for negatively charged phospholipids.

MATERIALS AND METHODS

Preparation of $(Na^+{+}K^+)$-ATPase

$(Na^+{+}K^+)$-ATPase from rabbit kidney outer medulla has been prepared according to the method described by Jørgensen (37). About 340 mg microsomes (on protein base) were incubated for one hour at 20° in a medium containing 0.58 mg/ml sodium dodecyl sulfate and 25 mM imidazole-HCl (pH 7.4), 3 mM ATP and 2 mM

EDTA (final protein concentration 1.45 mg protein per ml). After the extraction the microsomal suspension was centrifuged on a sucrose gradient (0-50%). The ATP of the pooled fractions of the gradient was removed by incubation at 37° in presence of Na^+, Mg^{2+} and K^+ and subsequent washing. The obtained membrane fragments, enriched in $(Na^+{+}K^+)$-ATPase, were stored in imidazole buffer (25 mM, pH 7.4) containing 10% sucrose. The specific $(Na^+{+}K^+)$-ATPase activity of the preparations ranged from 1.0 to 1.6 mmol P_i formed. mg^{-1} protein per h.

Preparation of liposomes

Liposomes have been prepared by a reversed phase evaporation method as described by Szoka and Papahadjopoulos (38). Mixtures of cholesterol, phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol and phosphatidylserine (ratio indicated in text and legends) in chloroform were evaporated under a stream of nitrogen to remove the organic solvent. After repeated washing with diethylether, a 1:1 mixture of diethylether and buffer solution of different composition was added and the solution thoroughly mixed on a vortex mixer, while the diethylether was again slowly evaporated by a stream of nitrogen. The final lipid content was 41 mg/ml. After all ether had disappeared the liposomes formed were sonicated for 30 min in a Branson sonicator bath at maximal output.

Determination of the vesicle volume

According to the method of Oku et al (39) 5 µl of a liposome suspension prepared in the presence of the fluorescent probe calcein (0.1 mM) was diluted with 1 ml buffer solution of equal osmolarity and the fluorescence was measured on a Shimadzu RP 510 spectrofluorimeter (exitation wave length 490, slit 10 nm; emission wavelength 520 nm, slit 10 nm) before (F_{tot}) and after (F_{in}) addition of 5 µl of a 10 mM $CoCl_2$ solution. Subsequently 25 µl of a 10% Triton X-100 solution in buffer was added and the fluorescence was measured again (F_{totq}). The trapped volume is calculated from $[F_{in} - (F_{totq} * r)]/[F_{tot} - (F_{totq} * r)] * 100 = \%$ trapped volume. In this formula the r stands for the dilution factor caused by addition of Triton X-100. Dilution due to addition of the $CoCl_2$ solution has been neglected.

Reconstitution procedure

Purified $(Na^+{+}K^+)$-ATPase (5 mg/ml) in 20 mM imidazole buffer (pH 7.2) was partially solubilized by incubation with cholate (final concentration 0.91% (w/v)) during 1 min at room temperature. Sucrose and bovine serum albumin were added to a final concentration of 20 and 5 mg/ml respectively. This mixture was then added to a ten-fold volume of liposomes, giving a lipid to protein ($(Na^+{+}K^+)$-ATPase) ratio of 85 (on weight basis). After thorough mixing, the preparation was frozen in liquid nitrogen or in a mixture of dry ice and acetone and subsequently thawed at room temperature. This freezing and thawing procedure was repeated twice. Thereafter the vesicle suspension was sonicated for 6 min in a Branson sonicator bath (at maximal output). Detergent was removed from the proteoliposomes by centrifuging aliquots of the suspension over a 10-fold volume Sephadex G-25 (coarse) column (equilibrated with the apropriate buffer solution) in a syringe. This centrifugation step took 5 min (100 g) and was repeated once. More than 99.9% of the cholate was removed by this procedure (40). Together with the removal of the detergent the extravesicular medium could be exchanged by a medium of choice (41).

ATP hydrolysis

The Na^+ and K^+ stimulated ATP hydrolysis was determined as the release of $^{32}P_i$ from [γ^{32}P] ATP (42). To 10 µl proteoliposomes containing Na^+, Mg^{2+} and K^+, in Tris buffer (pH 7.2), 190 µl of a medium containing Na^+, Mg^{2+}, K^+, Tris (pH 7.2), ouabain (0.2 mM) and 0.5 (or 1.0) mM labeled ATP were added at room temperature.

The Na^+ stimulated ATP hydrolysis of proteoliposomes without intravesicular K^+ was determined in a similar way. The extravesicular medium contained 1 (or 5) µM labeled ATP and was free of K^+. For blank values the reconstituted $(Na^+{+}K^+)$-ATPase was denatured with trichloroacetic acid prior to incubation with the assay medium. The $^{32}P_i$ production was measured after

stopping the reaction at a given time by addition of 0.4 ml 10% trichloroacetic acid followed by mixing with 0.4 ml 20% (w/v) aqueous charcoal suspension. The charcoal adsorbs the adenosine phosphates from the medium, but leaves P_i in solution. The suspension was mixed thoroughly during 10 s. This mixing was repeated twice after 5 and 10 min. Thereafter the charcoal was sedimented by centrifugation for 10 min at 2000 g at 0°. Aliquots (0.2-0.5 ml) were taken from the supernatant, mixed with 4.5 ml liquid scintillation fluid (Aqualuma Plus). Radioactivity was measured with a liquid scintillation counter.

Phosphorylation

Phosphorylation of the reconstituted $(Na^+ + K^+)$-ATPase was carried out at 22° at pH 7.0. The labeled ATP concentrations varied between 0.2 and 20 μM (The Radiochemical Centre, Amersham, UK., specific radioactivity 3000 Ci/mole). The reaction was started by rapid mixing of 10 μl proteoliposomes (preincubated with 0.2 mM ouabain and 2 mM Mg^{2+}, if not indicated otherwise) with 90 μl of the phosphorylation medium. The phosphorylation medium contained 100 mM Na^+, 1 mM Mg^{2+}, 0.1 mM EDTA, 30 mM Tris and labeled ATP. The reaction was stopped after 3 s by addition of 3 ml 5% (w/v) trichloroacetic acid, containing 100 mM phosphoric acid. The denatured phosphoprotein was filtered on a 1.2 μm pore width Selectron filter (Schleicher and Schuell, Dassel FRG), which was then washed three times with 3 ml of the stopping solution. Incorporated $^{32}P_i$ was determined by liquid scintillation counting. For blank values the proteoliposomes were mixed with the stopping solution prior to addition of ATP.

Materials

ATP and Tris were purchased from Boehringer, Mannheim, F.R.G.; calcein (fluorexon) was obtained from Aldrich, Steinheim, F.R.G.; [γ^{32}P]ATP was obtained from Amersham, Buckinghamshire, U.K. The phospholipids used in this study were either from Lipid Products, Nutfield Ridge, UK (phosphatidylserine) or from Avanti Polar Lipids, Birmingham AL, USA (all other phospholipids). The phospholipids were > 99% pure and the fatty acid composition was determined by gas chromatography of the methylated fatty acids after hydrolysis and methylation with BF_3 (43). The following phospholipids (with between parentheses source number and fatty acid composition were used: egg phosphatidylcholine(840051; 35% 16:0; 12% 18:0; 32% 18:1; 18% 18:2; 3% 20:4); egg phosphatidylethanolamine prepared by phospholipase D from egg phosphatidylcholine (841118; 38% 16:0; 11% 18:0; 32% 18:1; 16% 18:2; 2% 20:4); dioleoylphosphatidylethanolamine (850725; 100% 18:1); 1-palmitoyl,2-oleoyl-phosphatidic acid (840857; 48% 16:0: 52% 18:1); bovine liver phosphatidylinositol (840042; 3% 16:0; 55% 18:0; 5% 18:1; 5% 18:2; 14% 20:4; 12% 22:0; 3% 22:4; 3% 22:5); bovine brain phosphatidylserine (grade 1; 42% 18:0; 43% 18:1; 13% 20:0; 2% 22:0). Cholesterol was purchased from from Sigma, St. Louis, MO, U.S.A. All other chemicals were of reagent grade.

RESULTS

Lipid dependency of enzymatic properties

$(Na^+ + K^+)$-ATPase was reconstituted in lipid vesicles yielding a functionally active cation pump, which was tested by the capacity of the proteoliposomes to transport actively Rb^+ (44). The proteoliposomes also exhibited an ouabain insensitive phosphorylation reaction and ATPase activity upon addition of ATP to the medium indicating that the proteoliposomes contained inside-out oriented $(Na^+ + K^+)$-ATPase. $(Na^+ + K^+)$-ATPase was incorporated in proteoliposomes with a ratio of added lipids to protein of 85:1. With a phospholipid content of rabbit kidney of 0.6-0.8 mg/mg protein (21, 35, 37) the native phospholipids were diluted at least 100 fold.

The steady-state phosphorylation level and the rate of hydrolysis in the presence of ouabain and in the presence and absence of extracellular K^+ of reconstituted $(Na^+ + K^+)$-ATPase have been determined in proteoliposomes with different

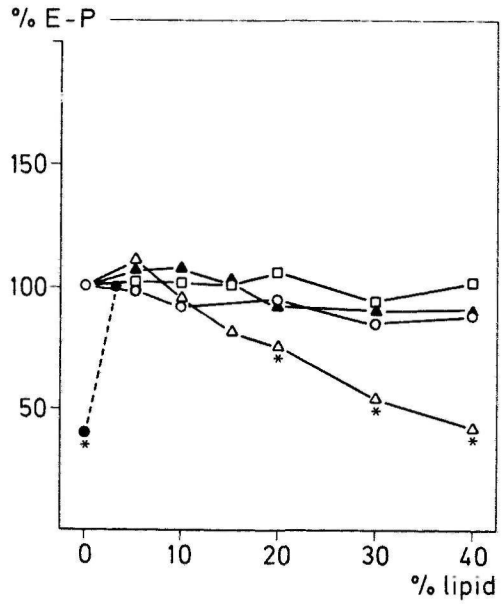

Figure 1. Number of phosphorylation sites of inside-out oriented (Na^++K^+)-ATPase in lipid vesicles with different lipid composition.

Prior to reconstitution (Na^++K^+)-ATPase was solubilized with cholate in the presence of 20 mg/ml sucrose and 5 mg/ml bovine serum albumin exept for the series of proteoliposomes in which cholesterol was varied (•). (The data of cholesterol concentrations above 5% coincided with the results of proteoliposomes with variable cholesterol concentrations reconstituted in the presence of sucrose and bovine serum albumin (o) and are not shown.) The intravesicular medium of the proteoliposomes consisted of 100 mM Na^+, 1 mM Mg^{2+}, 0.1 mM EDTA and 30 mM Tris (pH 7.0). The Na^+ of the extravesicular medium was replaced by Tris during the gel filtration step of the reconstitution procedure, prior to incubation of the proteoliposomes with 0.2 mM ouabain during one hour at room temperature. Immediately thereafter the phosphorylation reaction was carried out as described in Materials and Methods in the presence of 1 μM labeled ATP. The series of proteoliposomes in which one lipid was varied were prepared in parallel. The phosphorylation levels of the proteoliposomes in each series were normalized with respect to those consisting of 0% of the lipid of interest, 39 mg/ml phosphatidylcholine and 2 mg/ml cholesterol (except for (•)), since the variation between series was larger than that between points in one series. The 100% values correspond to phosphorylation levels of 250-500 pmole per mg protein. The values are the mean of six independent experiments carried out in duplicate. The symbols represent the phosphorylation level of proteoliposomes in which the following compounds were varied: (o) cholesterol, (•) cholesterol (reconstitution in the absence of sucrose and bovine serum albumin), (□) phosphatidylethanolamine, (△) phosphatidic acid and (▲) phosphatidylserine. Data points significantly ($p < 0.05$) different from 100% are indicated with an asterisk (*).

lipid composition From these parameters the molar activity was derived and taken as the parameter for the activity of the enzyme in a different lipid environment

The steady-state phosphorylation level

In order to determine the number of active inside-out oriented enzyme molecules the steady-state phosphorylation level was measured under optimal phosphorylation conditions In order to exclude enzyme molecules, which were not properly incorporated, the proteoliposomes were preincubated with ouabain In this way the enzyme molecules, which have no defined interaction with the lipids in the liposomes (not incorporated), were blocked by the inhibitor The rightside-out oriented pump molecules cannot be phosphorylated since ATP cannot penetrate the proteoliposome and ouabain blocks the not-incorporated enzyme molecules The inside-out oriented (Na^++K^+)-ATPase molecules cannot bind ouabain since they have the ouabain binding site intravesicularly and are the only population which can be phosphorylated under these conditions

The intravesicular medium (extracellular with respect to the inside-out oriented molecules) was the same as the phosphorylation medium except for the absence of ATP) During the preincubation with ouabain, Na^+ in the extravesicular medium was replaced by Tris in order to avoid the decrease of the ouabain binding by Na^+

The phosphorylation level was first determined in proteoliposomes prepared of liposomes containing egg phosphatidylcholine as the only lipid compound These proteoliposomes yielded a very low phosphorylation level Replacement of part of the phosphatidylcholine by small amounts of cholesterol increased the steady-state phosphorylation level markedly This effect occurred already at the lowest *concentration of cholesterol tested (2 mg/ml) Further replacement of phosphatidylcholine by cholesterol did not increase the phosphorylation level Cholesterol amounts of more than 20% of the total lipid (41 mg/ml) slightly decreased the steady-state phosphorylation level When sucrose (20 mg/ml) and bovine serum albumin (5 mg/ml) were present during the reconstitution procedure the phosphorylation level was much higher than in the absence of these additives In the presence of sucrose and bovine serum albumin cholesterol at low concentrations only slightly decreased the number of phosphorylation sites (Fig 1) When the sonication step was left out of the reconstitution procedure no increase of the phosphorylation level was caused by cholesterol This indicates that the low phosphorylation level in the absence of cholesterol was caused by an inactivation of the enzyme during the sonication step Cholesterol apparently can protect the enzyme against this inactivation Sucrose and bovine serum albumin can (partially) take over this protective role of cholesterol

In subsequent experiments a lipid mixture of 39 mg/ml phosphatidylcholine and 2 mg/ml cholesterol was taken together with 5 mg/ml bovine serum albumin and 20 mg/ml sucrose in order to obtain the optimal phosphorylation level (In control experiments the cholesterol was left out, which did not change the results with respect to the influence of phospholipid composition on the enzyme activity) Starting with this lipid composition phosphatidylcholine was replaced in part by the phospholipids of interest in a series of proteoliposomes The number of phosphorylation sites of inside-out oriented (Na^++K^+)-ATPase in proteoliposomes containing different concentrations of two negatively charged phospholipids (phosphatidylserine and phosphatidic acid) and the neutral phosphatidylethanolamine have been determined The neutral phosphatidylethanolamine with the same fatty acid composition as egg phosphatidylcholine did not change the number of phosphorylation sites significantly when it replaced phosphatidylcholine in the reconstituted system (Fig 1) The presence of phosphatidylserine did not change the number of phosphorylation sites significantly at the concentrations tested (Fig 1) Phosphatidylinositol neither influenced the number of phosphorylatable sites (not shown) The presence of the negatively charged phospholipid phosphatidic acid decreased the steady-state phosphorylation level significantly when the phosphatidic acid content increased above 10%

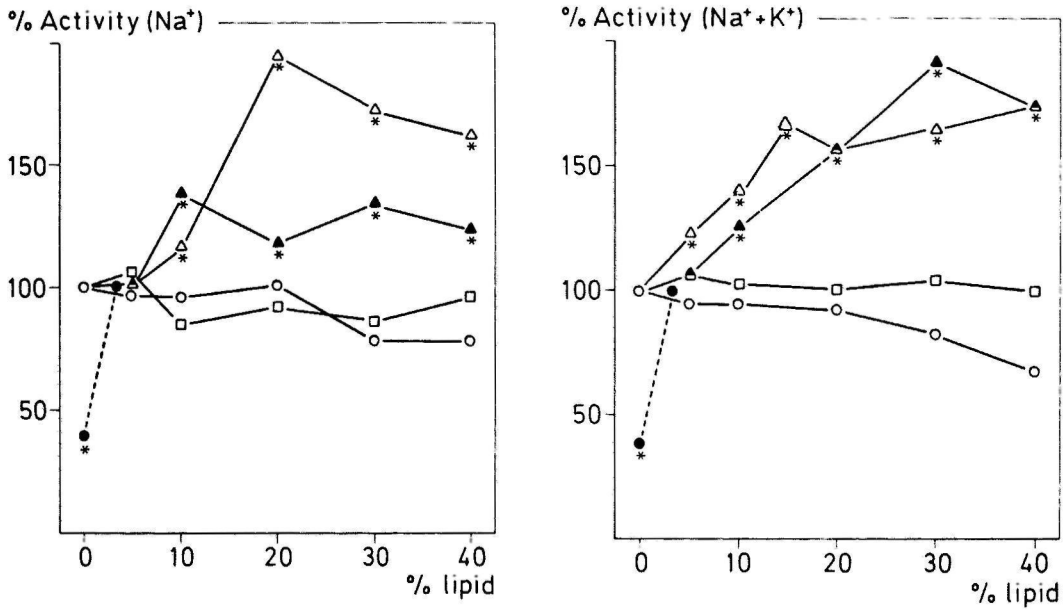

Figure 2. (left) Lipid dependency of the Na^+-stimulated ATPase activity of inside-out incorporated (Na^++K^+)-ATPase reconstituted in lipid vesicles.

The hydrolytic activity of the proteoliposomes of Fig. 1 was determined at 22°C during 10 and 20 s in the phosphorylation medium containing 1 μM labeled ATP. The production of P_i was determined as described in Materials and Methods. Normalization was the same as the same as in Fig. 1. The values are the mean of three independent experiments carried out in duplicate. The 100% values correspond to hydrolytic activities of 1-2 μmole P_i formed per mg protein per hour. The symbols represent the Na^+-ATPase activity of proteoliposomes in which the following compounds were varied: (o) cholesterol, (•) cholesterol (reconstitution in the absence of sucrose and bovine serum albumin), (□) phosphatidylethanolamine, (△) phosphatidic acid and (▲) phosphatidylserine. Data points significantly ($p < 0.05$) different from 100% are indicated with an asterisk (*).

Figure 3. (right) Lipid dependency of the Na^++K^+-stimulated ATPase activity of inside-out incorporated (Na^++K^+)-ATPase reconstituted in lipid vesicles.

The hydrolytic activity of proteoliposomes as in Fig. 1 in which 5 mM of the intravesicular Na^+ was replaced by 5 mM K^+ was determined by incubation at 22°C during 30 and 60 s in the presence of 95 mM Na^+, 5 mM K^+, 0.1 mM EDTA, 1 mM Mg^{2+} and 0.5 mM labeled ATP. The P_i production was determined as described in Materials and Methods. Normalization was the same as in Fig. 1. The values are the mean of six independent experiments carried out in duplicate. The 100% values correspond to hydrolytic activities of 30-60 μmole P_i formed per mg protein per hour. The symbols represent the hydrolytic activity of (Na^++K^+)-ATPase in proteoliposomes in which the following lipid compounds were varied: (o) cholesterol, (•) cholesterol (reconstitution in the absence of sucrose and bovine serum albumin), (□) phosphatidylethanolamine, (△) phosphatidic acid and (▲) phosphatidylserine. Data points significantly ($p < 0.05$) different from 100% are indicated with an asterisk (*).

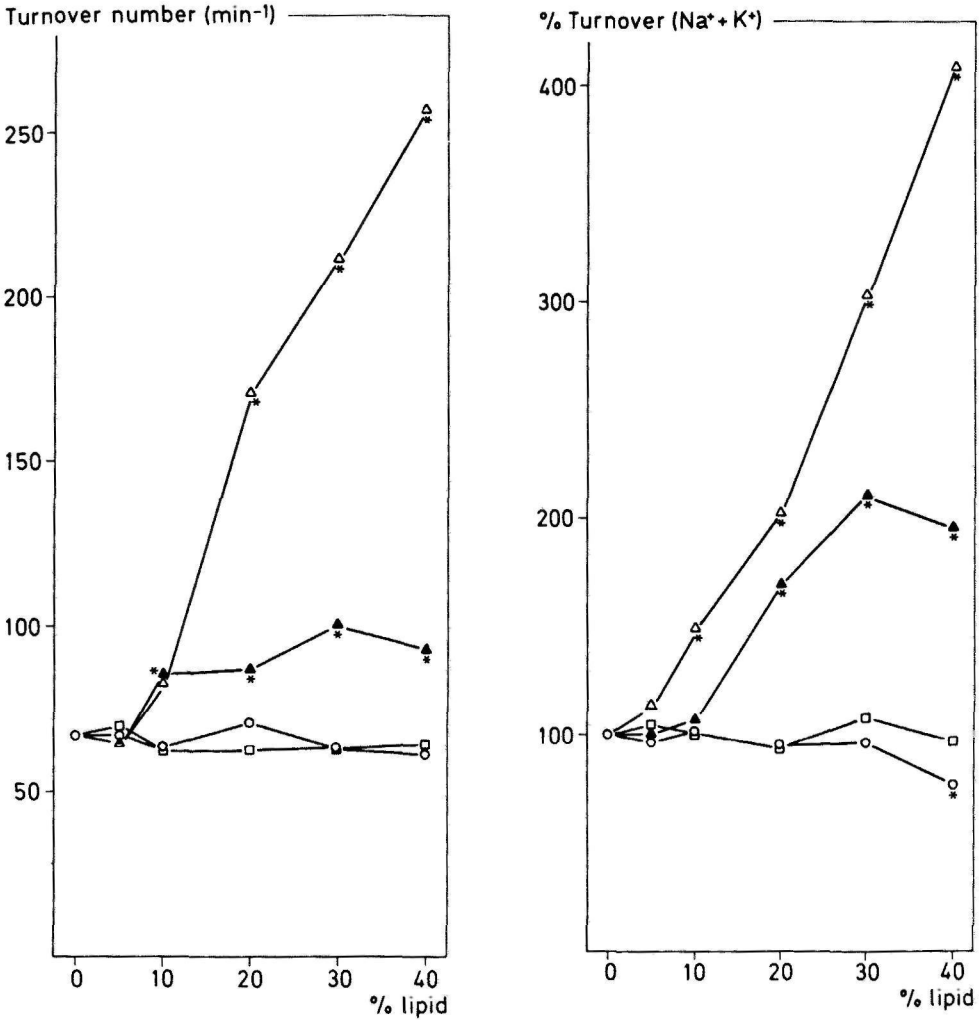

Figure 4. (left) Lipid dependency of the Na^+-stimulated turnover number of inside-out incorporated (Na^++K^+)-ATPase reconstituted in lipid vesicles.
From the ratio of the Na^+-ATPase activity (Fig. 2) and the number of the inside-out oriented (Na^++K^+)-ATPase molecules the turnover number of the proteoliposomes was calculated. The values represent the mean of three independent experiments in which the hydrolytic activity and number of phosphorylation site were determined from the same proteoliposome batch. The symbols represent the turnover number of inside-out reconstituted (Na^++K^+)-ATPase in lipid vesicles in which the following lipids were replaced: (o) cholesterol, (□) phosphatidylethanolamine, (△) phosphatidic acid and (▲) phosphatidylserine. Data points significantly ($p < 0.05$) different from 100% are indicated with an asterisk (*).

Figure 5. (right) Lipid dependency of the Na^++K^+-stimulated turnover number of inside-out incorporated (Na^++K^+)-ATPase reconstituted in lipid vesicles.
From the ratio of the (Na^++K^+)-ATPase activity (Fig. 3) and the number of the inside-out oriented (Na^++K^+)-ATPase molecules (Fig. 1) the molar activity of the proteoliposomes was calculated. The molar activity of the proteoliposomes consisting of 39 mg/ml phosphatidylcholine and 2 mg/ml cholesterol were set at 100%. The other values were normalized with respect to this value. The 100% value represents a molar activity of 2000 ± 300 min^{-1} (mean ± S.D.). The symbols represent the molar activity of inside-out reconstituted (Na^++K^+)-ATPase in lipid vesicles in which the following lipids were replaced: (o) cholesterol, (□) phosphatidylethanolamine, (△) phosphatidic acid and (▲) phosphatidylserine. Data points significantly ($p < 0.05$) different from 100% are indicated with an asterisk (*).

(Fig 1) Phosphorylation experiments with 10 μM ATP instead of 1 μM yielded similar results (not shown)

Na^+ stimulated hydrolysis and turnover number

ATP-hydrolysis of the proteoliposomes was measured during 10 and 20 s under the same conditions as the phosphorylation reaction The lipid dependency of the Na^+-ATPase activity of the reconstituted (Na^++K^+)-ATPase was similar to that of the number of phosphorylation sites with respect to the the cholesterol content of the proteoliposomes This resulted in a turnover number being independent of the cholesterol concentration of the proteoliposome (Figs 2 and 4) Replacement of phosphatidylcholine by the neutral phospholipid phosphatidylethanolamine did not change the rate of hydrolysis either Since the phosphatidylethanolamine content had neither an effect on the number of phosphorylation sites nor on the Na^+-ATPase activity the turnover number was not influenced by this phospholipid. The negatively charged phosphatidic acid enhanced the rate of hydrolysis at all concentrations tested, whereas phosphatidylserine increased this rate at concentrations higher than 5% This increase also resulted in the case of phosphatidylserine in an enhancement of the turnover number at concentrations above 5% Together with a lowering of the steady-state phosphorylation level at higher concentrations this resulted in a significant increase of the turnover number of the enzyme for phosphatidic acid at concentrations above 5% (Figs 2 and 4)

Na^+ and K^+ stimulated hydrolysis and molar activity

In order to determine the rate of Na^+ and K^+ stimulated hydrolyis of the inside-out oriented (Na^++K^+)-ATPase the proteoliposomes were prepared with intravesicular K^+ in addition to the content of the vesicles used in the phosphorylation experiments (5 mM of the Na^+ was replaced by 5 mM K^+ in the medium in which the liposomes were prepared) To avoid membrane potentials caused by ion gradients the extravesicular medium was chosen to be the same as the intravesicular medium except for the presence of ATP in the extravesicular medium The (Na^++K^+)-ATPase activity showed a qualitatively similar dependence on the lipid composition of the proteoliposomes as the hydrolytic activity in the absence of K^+ Replacement of phosphatidylcholine by low concentrations of cholesterol in the proteoliposomes did not influence the K^+ stimulated activity, provided that bovine serum albumin and sucrose were present Cholesterol decreased the rate of hydrolysis slightly at concentrations of 30 and 40% (Fig 3) This resulted in a molar activity which was hardly dependent on the cholesterol concentration (Fig 5) Phosphatidylethanolamine had no effect on the rate of hydrolysis resulting in a molar activity which was not influenced by the phosphatidylethanolamine concentration (Figs 3 and 5) Phosphatidylserine, phosphatidic acid (and phosphatidylinositol, not shown) increased the rate of hydrolysis at all concentrations tested (Fig 3), resulting in significant increase of the molar activities at increasing concentrations of the negatively charged phospholipids (Fig 5)

Lipid dependence of the orientation and incorporation of (Na^++K^+)-ATPase

In order to determine whether the change in the steady-state phosphorylation level was either caused by the degree of inactivation of the enzyme during the reconstitution procedure or was a consequence of the differences in the number of inside-out incorporated enzyme molecules, the ratio of inside-out rightside-out not incorporated pump molecules in proteoliposomes with different lipid composition was determined with the ouabain/cholate method (24) To obtain the amount of inside-out oriented pump molecules the reconstituted (Na^++K^+)-ATPase was phosphorylated with ATP after preincubation with ouabain and Mg^{2+} The not-incorporated part of the pump molecules was blocked by the inhibitor and the rightside-out part could not be phosphorylated since ATP cannot penetrate the vesicles In the absence of ouabain the not incorporated ATPase molecules were phosphorylated as well Disturbing the bilayer integrity with a detergent made the vesicles permeable for ATP Phosphorylation of

the detergent treated proteoliposomes in the absence of ouabain yielded a figure for the total of $(Na^+ + K^+)$-ATPase populations inside-out+rightside-out+not-incorporated. Treatment with detergents of the proteoliposomes did, however, not only lead to permeable vesicles but also resulted in a concentration dependent inactivation of the enzyme

Using cholate the inactivation of the enzyme and the opening of the vesicles were tested in separate experiments The opening of the vesicles by detergents was studied with the method of Oku et al (39) By titrating with a detergent in the cuvette the minimal concentration necessary for complete opening of the vesicles could be determined For cholate this appeared to be 0 4% (w/v) for vesicles of all lipid compositions The minimal concentration of cholate causing inactivation of the enzyme in the presence of added lipids was 0 5% So to open the proteoliposomes a small concentration range in the proximity of these two concentrations (0 3-0 6%) was chosen The optimum phosphorylation level at that detergent concentration was taken as a figure for the sum of the inside-out, rightside-out and not-incorporated enzyme

The fraction of inside-out was not influenced by the lipid composition of the proteoliposomes tested above except when phosphatidic acid was used A decrease of with a maximum of 41% in the number of inside-out oriented $(Na^+ + K^+)$-ATPase molecules in parallel with a relative increase of the rightside-out and not-incorporated part of the population was observed by increasing concentrations of phosphatidic acid The deviation with the other lipids was always below 10%

Determination of the intactness of the liposomes

The activity of the incorporated $(Na^+ + K^+)$-ATPase in the proteoliposomes determined in the presence of ouabain is dependent on the impermeability of the vesicles In order to check whether the proteoliposomes were tight the volumes enclosed by the liposomes were determined by the method of Oku et al (39) When the vesicles were not tight they might either lose calcein from the interior which would be quenched by exterior $CoCl_2$ or the fluorescence of the intravesicular calcein was quenched by the $CoCl_2$ penetrating the vesicle Therefore, the calcein fluorescence gave an indication for the leakiness of the proteoliposomes With leaky vesicles the number of inside-out incorporated $(Na^+ + K^+)$-ATPase molecules may be underestimated since ouabain may penetrate the vesicle and block pump molecules with this orientation Leakiness of the vesicles may also lead to overestimation of the inside-out oriented population in the absence of ouabain since ATP may penetrate the vesicles and phosphorylate $(Na^+ + K^+)$-ATPase molecules with their ATP binding site at the vesicle interior

The volume of the phosphatidylcholine containing liposomes determined with the calcein fluorescence was 18 ± 1 5% (mean ± SD, n=5) of the total volume This volume was hardly dependent on the lipid composition of the liposomes the volume of vesicles containing 40% cholesterol, phosphatidic acid, phosphatidylethanolamine, or phosphatidylserine were 17, 20, 15 and 20% of the total volume, respectively This finding indicates that the integrity was not changed by a variation in the lipid composition

DISCUSSION

In the dispute on the lipid dependency of $(Na^+ + K^+)$-ATPase no clear view concerning the need for specific lipids has emerged so far It is clear that membrane bound enzymes like $(Na^+ + K^+)$-ATPase need a lipid bilayer for their function The need for specific lipids necessary for the full activity of the ATPase, however is still not clear

In most studies delipidated and inactivated enzyme is relipidated with pure phospholipids and the recovery of the enzyme activity is taken as measure for the need for a specific phospholipid Alternatively, the native lipids of the enzyme are either enzymatically removed or modified The disadvantage of the first method is that the effect of the delipidation procedure on the enzyme is

unknown and that the reactivation by a specific lipid is dependent on the delipidation procedure, the detergent used and the accompanying inactivation of the enzyme (45). Another disadvantage of this method is that the nature of the interaction of the added lipids with the protein after delipidation is unknown since it is not sure that the lipids surround the enzyme in a way comparable to the natural state. The disadvantage of the enzymatic method is the possibility of a side effect by either impurities in the enzyme preparation or breakdown products of the substrate.

In this study partially solubilized (Na^++K^+)-ATPase was incorporated in liposomes of known lipid composition and the turnover of the inside-out oriented pump molecules was determined. Since the proteoliposomes were competent of cation transport (44) it is obvious that the protein must span the bilayer. In this situation it is likely that the interaction of the protein with the lipids surrounding it in the proteoliposomes is similar to that in the in vivo situation. The native lipids were not removed during the solubilization and reconstitution procedure but are merely diluted by the added lipids. Based on a lipid content of the purified (Na^++K^+)-ATPase of 0.6 to 0.8 mg/mg protein (21, 35, 37) the amount of native lipids is only about 1% of the total lipids. The observation that the lipids of the annulus surrounding (Na^++K^+)-ATPase do exchange with the lipids of the bilayer (46) does not support the concept of long-lived boundary of annular layers of lipids (47). There is a preference for negatively charged lipids in the direct proximity of the enzyme (46, 48) probably due to the interaction of the lipid with the positively charged amino groups in the boundary layer of the enzyme (49). This exchange, however makes the lipid environment strongly dependent on the lipid composition of the proteoliposomes.

In this study the fatty acid composition of the lipids used is not the same for all lipids. In a study on the effects on the transport rate of reconstituted (Na^++K^+)-ATPase it was shown that that the fatty acid composition of the proteoliposomes played a only minor role in the turnover rate of the enzyme (50). In the present study control experiments with synthetic dioleoylphosphatidylethanolamine instead of phosphatidylethanolamine with the same fatty acid composition as egg phosphatidylcholine have been carried out and no differences were observed.

Orientation of the reconstituted (Na^++K^+)-ATPase

It has been reported that the orientation of the reconstituted enzyme can be influenced by the lipid composition of the proteoliposomes (24). This is a disadvantage of this method when a parameter (e.g. the steady-state phosphorylation level) is determined as an absolute measure for the recovery of the enzyme activity after reconstitution. When for instance there is a shift towards inside-out incorporation an increase in the phosphorylation level will be observed. With the ouabain/cholate method (24) the orientation and the amount of incorporation were determined. With phosphatidylethanolamine and phosphatidylserine no differences in the degree of orientation was observed. When phosphatidic acid was used a concentration dependent change in the orientation occurred, which may only partially explain the reduction of the number of phosphorylation sites, since this effect was relatively small. However, since only inside-out oriented enzyme molecules can be phosphorylated and hydrolyse ATP, a change in the orientation has no effect on the molar activity.

Lipid dependency of the enzymatic parameters

Since in the absence of K^+ the rate of phosphorylation is much faster than the rate of dephosphorylation (51, 52) the amount of phosphoenzyme formed from [γ-^{32}P]ATP equals the number of phosphorylatable sites. Since the chosen conditions for the phosphorylation reaction were optimal (Na^+ and Mg^{2+} were present in saturating concentrations) and the phosphorylation level did not change at higer ATP concentrations (not shown) the determined steady-state phosphorylation level was taken as a measure for the number of inside-out oriented (Na^++K^+)-ATPase molecules. Under these optimal conditions almost all of the (Na^++K^+)-ATPase will be in the

phosphorylated state since it has been reported that the phosphorylation reaction is not much dependent on the lipid environment (53, 54) The activity of the inside-out oriented enzyme molecules in the absence and presence of K^+ was determined and the turnover number as a result of the ratio of this parameter and the number of the inside-out oriented pump molecules The latter was determined by the number of phosphorylation sites as a parameter for catalytically active enzyme molecules

It has been observed that cholesterol at higher concentrations decreased the phosphorylation level in parallel with the hydrolytic activity at higher concentrations and did not influence the turnover rate Cholesterol is therefore not of great importance for the enzyme activity which finding is in accordance with results of Peters et al (35) and Wheeler and Isern De Caldentey (34), but in conflict with results of Järnefelt (27), Noguchi and Freed (28) and Seiler and Fiehn (29) It is possible that during the removal of cholesterol the enzyme is reversibly inactivated and that the reactivation by cholesterol is dependent on the procedure of delipidation This was found by us using low concentrations of cholesterol An increase of the phosphorylation level was observed at low concentrations of cholesterol compared to proteoliposomes with phosphatidylcholine as the only lipid When bovine serum albumin and sucrose were added to the reconstitution mixture or when the sonication time was reduced the phosphorylation level was less or not at all influenced by low concentrations of cholesterol It was therefore concluded that low concentrations of cholesterol only protected the enzyme against inactivation during the reconstitution procedure In order to obtain a reasonable phosphorylation level under all conditions a minor amount of cholesterol of 2 mg/ml was always present in the proteoliposomes With a somewhat changed reconstitution procedure in which the sonication step was left out the phospholipid dependency of the turnover of reconstituted $(Na^+{+}K^+)$-ATPase was determined in the absence of added cholesterol These results agreed qualitatively with the experiments described above

The neutral phospholipid phosphatidylethanolamine neither influenced the rate in the absence of K^+ nor enhanced the Na^+ and K^+ stimulated ATPase activity The negatively charged phospholipids phosphatidic acid, phosphatidylserine and phosphatidylinositol increased the turnover rate both in the presence and in the absence of K^+ This supports results of several groups who reported higher activities in the presence of negatively charged phospholipids Qualitatively the activation of the three negatively charged phospholipids can be well compared Quantitatively some differences exist between the three negatively charged lipids The best reactivation seems to be obtained in the series phosphatidic acid> phosphatidylserine> phosphatidylinositol

The calculated molar activity of the $(Na^+{+}K^+)$-ATPase reconstituted in vesicles containing 39 mg/ml phosphatidylcholine and 2 mg/ml cholesterol was about 2000 min^{-1} at 22°C Since the molar activity of the purified enzyme under identical conditions was about 3000 min^{-1}, it may be concluded that the new lipid environment reduced this activity When phosphatidylinositol was present in the proteoliposomes the molar actitvity of the reconstituted enzyme increased to the same value as the purified enzyme With phosphatidylserine and phosphatidic acid present in the proteoliposomes the molar activity increased to about 4000 and 8000 min^{-1} respectively These values are far above that of the purified enzyme, indicating that the native lipid environment is not optimal for activation of the reaction cycle of $(Na^+{+}K^+)$-ATPase

The reactivation by phosphatidylserine cannot be due to the chelating effect of this phospholipid, as proposed by Specht and Robinson (55), since the experiments were all carried out in the presence of 0 2 mM EDTA In the light of a proposal of Hegyvary et al (54) that lipids enhance the conformational change from E_1P to E_2P it may be considered that negatively charged lipids exert this stimulation more effectively than uncharged phospholipids This might be due to stronger interaction of negatively charged phospholipids with the protein via positively charged amine groups of the protein (48) The observation that the Na^+ plus K^+ activated molar activity is more

enhanced than that stimulated by Na^+ alone indicates that phospholipids also play a role in the reaction cycle in steps subsequent to the dephosphorylation reaction Study on the effects of phospholipids on the partial reactions is required to elucidate the mechanism of the described effects of phospholipids on the enzyme activity.

It can, however be concluded that the presence of negatively charged phospholipids is no absolute requirement for the activity of $(Na^+ + K^+)$-ATPase since in the presence of phosphatidylcholine as the only lipid compound the reconstituted $(Na^+ + K^+)$-ATPase is also active.

Since the recovery of the enzyme strongly depends on the method used for delipidation and reconstitution (3, 20, 27), the restoration of the activity itself seems to be no suitabele parameter to study the lipid dependency of a membrane bound enzyme The molar activity of the purified enzyme incorporated in proteoliposomes provides a better system

It can be concluded that cholesterol has no effect on the phosphorylation level and molar activity of the enzyme and that negatively charged phospholipids enhance the molar activity, but that they are no prerequisite for the activity of $(Na^+ + K^+)$-ATPase

Acknowledgements

This investigation was financially supported by the Netherlands Organization for Scientific Research (N W O), through the Netherlands Biophysics Foundation Mr H G P Swarts is gratefully acknowledged for the fatty acid analysis of the phospholipids

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Cation sidedness in the phosphorylation step of Na^+K^+ ATP-ase.

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Biochim. Biophys. Acta 983 (1989) 142-152

Biochimica et Biophysica Acta, 983 (1989) 142–152
Elsevier

BBAMEM 74506

Cation sidedness in the phosphorylation step of Na^+/K^+-ATPase

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(Received 24 February 1989)

Key words ATPase, Na^+/K^+-, Cation sidedness, Phosphorylation, Liposome

Na^+/K^+-ATPase, reconstituted into phospholipid vesicles, has been used to study the localisation of binding sites of ligands involved in the phosphorylation reaction. Inside-out oriented Na^+/K^+-ATPase molecules are the only population in this system, which can be phosphorylated, as the rightside-out oriented as well as the non-incorporated enzyme molecules are inhibited by ouabain. In addition, the rightside-out oriented Na^+/K^+-ATPase molecules have their ATP binding site intravesicularly and are thus not accessible to substrate added to the extravesicular medium. Functional binding sites for the following ligands have been demonstrated: (i) Potassium, acting at the extracellular side: with high affinity (stimulating the dephosphorylation rate of the E_2P conformation) and low affinity (inducing the non-phosphorylating E_2K complex). (ii) Potassium, acting at the cytoplasmic side with both high and low affinity. The latter sites are also responsible for the formation of an E_2K complex and compete with Na^+ for its binding sites. (iii) Sodium at the cytoplasmic side responsible for stimulation of the phosphorylation reaction. (iv) Sodium (and amine buffers) at the extracellular side enhancing the phosphorylation level of Na^+/K^+-ATPase where choline chloride has no effect. (v) Magnesium at the cytoplasmic side, stimulating the phosphorylation reaction and inhibiting it above optimal concentrations.

Introduction

In the mechanism of action of Na^+/K^+-ATPase the phosphorylation step plays a pivotal role [1–5] It is assumed to be a crucial step in the conversion of chemical energy into transport Nearly all studies on the phosphorylation reaction have been carried out in membrane preparations in which added ligands have simultaneous access to binding sites located at the extracellular and the cytosolic side of the plasma membrane In those studies it has been established that a maximal level of phosphorylation can be obtained in the presence of both Mg^{2+} and Na^+ and in the absence of K^+ ions The latter ion stimulates the rate of the dephosphorylation reaction considerably.

However, the presence of ions at both sides of the membrane precludes conclusions on the sidedness of these effects Although it is very likely that the stimulatory effects of Na^+ on the phosphorylation reaction take place at the cytosolic and the stimulatory effects of K^+ on the dephosphorylation reaction at the extracellular side, additional effects of these ions at the opposite sides cannot be excluded Although Mg^{2+} is supposed to be involved in the binding of phosphate at the cytosolic side, possible effects at the extracellular side have not been studied before

We therefore studied steady-state phosphorylation of Na^+/K^+-ATPase reconstituted in proteoliposomes, in which only phosphorylation of inside-out oriented pump molecules is possible By variation of the ion composition in- and outside the vesicles insight has been obtained on the presence, location and function of ion-binding sites on Na^+/K^+-ATPase

Materials and Methods

Preparation of Na^+/K^+-ATPase

Na^+/K^+-ATPase from rabbit kidney outer medulla has been prepared according to the method described by Jørgensen [6] About 340 mg microsomes (on protein base) were incubated for one hour at 20 °C in a medium containing 0 58 mg/ml sodium dodecyl sulfate and 25 mM imidazole-HCl (pH 7.4), 3 mM ATP and 2 mM EDTA (final protein concentration 145 mg protein per ml) After the extraction the microsomal suspension was centrifuged on a sucrose gradient (0–50%) The ATP of

Abbreviations Tris, tris(hydroxymethyl)aminomethane, EDTA, ethylenediaminetetraacetic acid, CCCP carbonylcyanide *m*-chlorophenylhydrazone K_{cyt}, Na_{cyt} and Mg_{cyt} represent K^+, Na^+ and Mg^{2+} concentrations at the cytosolic side respectively K_{ext} Na_{ext} and Mg_{ext} represent these concentrations at the extracellular side

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the pooled fractions of the gradient was removed by incubation at 37°C in presence of Na^+, Mg^{2+} and K^+ and subsequent washing The obtained membrane fragments, enriched in Na^+/K^+-ATPase were stored in imidazole buffer (25 mM, pH 7 4) containing 10% sucrose The specific Na^+/K^+-ATPase activity of the preparations ranged from 1 0 to 1 6 mmol P_i formed per mg protein per h

Preparation of liposomes

Liposomes have been prepared by a reversed phase evaporation method as described by Szoka and Papahadjopoulos [7] Mixtures of cholesterol, phosphatidylcholine and phosphatidylserine (ratio indicated in text and legends) in chloroform were evaporated under a stream of nitrogen to remove the organic solvent After repeated washing with diethyl ether, a 1 1 mixture of diethyl ether and buffer solution of different composition was added and the solution thoroughly mixed on a vortex mixer, while the diethyl ether was again slowly evaporated by a stream of nitrogen The final lipid content was between 20 and 50 mg/ml After all diethyl ether had disappeared the liposomes formed were sonicated for 30 min in a Branson sonicator bath at maximal output

Reconstitution procedure

Purified Na^+/K^+-ATPase (5 mg/ml) in 20 mM imidazole buffer (pH 7 2) was partially solubilized by incubation with cholate (final concentration 0 91% w/v) during 1 min at room temperature This mixture was then added to a 10-fold volume of liposomes, giving a lipid to protein ratio of 40 to 100 (on weight basis) After thorough mixing, the preparation was frozen in liquid nitrogen or in a mixture of dry ice and acetone and subsequently thawed at room temperature This freezing and thawing procedure was repeated twice Thereafter the vesicle suspension was sonicated for 6 min in a Branson sonicator bath (at maximal output) Detergent was removed from the proteoliposomes by centrifuging aliquots of the suspension over a 10-fold volume Sephadex G-25 (coarse) column (equilibrated with the appropriate buffer solution) in a syringe This centrifugation step took 5 min (100 g) and was repeated once More than 99 9% of the cholate was removed by this procedure [8] Together with the removal of the detergent the extravesicular medium could be exchanged by a medium of choice [9]

ATP hydrolysis

The ATP hydrolysis was determined as the release of $^{32}P_i$ from [γ-^{32}P] ATP [10] To 10 μl proteoliposomes containing Na^+, Mg^{2+} and K^+, in Tris buffer (pH 7 2), 190 μl of a medium containing Na^+, Mg^{2+}, K^+, Tris (pH 7 2), ouabain (0 2 mM) and 0 1 to 1 0 mM labeled ATP were added at room temperature For blanc values the reconstituted Na^+/K^+-ATPase was denatured with trichloroacetic acid prior to incubation with the assay medium The $^{32}P_i$ production was measured after stopping the reaction at a given time by addition of 0 4 ml 10% trichloroacetic acid followed by mixing with 0 4 ml 20% (w/v) aqueous charcoal suspension The charcoal adsorbs the adenosine phosphates from the medium but leaves P_i in solution The suspension was mixed thoroughly during 10 s each 5 min (three times) Thereafter the charcoal was sedimented by centrifugation for 10 min at 2000 × *g* at 0°C Aliquots (0 2–0 5 ml) were taken from the supernatant, mixed with 4 5 ml liquid scintillation fluid (Aqualuma Plus) Radioactivity was measured with a liquid scintillation counter

Transport assay

For transport studies proteoliposomes loaded with 20 mM K^+ were equilibrated with a medium containing $^{86}Rb^+$ (0 1 mM) during 3 h at room temperature [11] After loading with the tracer the proteoliposome suspension was incubated with a 10-fold volume of transport medium containing Na^+, K^+, Mg^{2+} and ouabain with or without ATP After the incubation at room temperature the transport was quenched by layering an aliquot of the suspension on a Dowex 50-X8 (Tris-form) column [12] The proteoliposomes were then eluted with 1 ml of a solution of 250 mM sucrose and 25 mg/ml bovine serum albumin The eluate containing proteoliposomes devoid of the external Rb^+, was counted in a liquid scintillation counter by measuring Cerenkov radiation

Phosphorylation

Phosphorylation of the reconstituted Na^+/K^+-ATPase was carried out at 22°C at pH 7 0 The labeled ATP concentrations varied between 0 2 and 20 μM (The Radiochemical Centre Amersham, U K , specific radioactivity 3000 Ci/mol) The reaction was started by rapid mixing of 10 μl proteoliposomes (preincubated with 0 2 mM ouabain and 2 mM Mg^{2+} if not indicated otherwise) with 90 μl of the medium containing ATP and the other ligands The reaction was stopped after 3 s by addition of 3 ml 5% (w/v) trichloroacetic acid containing 100 mM phosphoric acid The denatured phosphoprotein was filtered on a 1 2 μm pore width Selectron filter (Schleicher and Schuell Dassel, F R G) which was then washed three times with 3 ml of the stopping solution Incorporated $^{32}P_i$ was determined by liquid scintillation counting For blanc values the proteoliposomes were mixed with the stopping solution prior to addition of ATP

Dephosphorylation

After phosphorylation for 10 s at room temperature 900 μl of the dephosphorylation medium was added to the phosphorylation mixture (100 μl) The dephospho-

rylation mixture contained, apart from the buffer and cations, 1 mM unlabeled ATP in order to dilute the labeled ATP 1000-fold. Together with the 10-fold dilution of the volume of the medium the final dilution of the labeled ATP was 10000 which actually avoided further phosphorylation by the labeled ATP. After rapid mixing the dephosphorylation reaction was stopped (at the time indicated) by addition of 5 ml 5% (w/v) trichloroacetic acid containing 100 mM phosphoric acid. After stopping the reaction, the mixture was further treated as described in the phosphorylation procedure.

Materials

ATP and Tris were purchased from Boehringer, Mannheim, F.R.G. [γ-^{32}P]ATP and ^{86}Rb were obtained from Amersham, Buckinghamshire, U.K., phosphatidylcholine (egg) and phosphatidylserine (bovine brain) were purchased from Avanti Polar Lipids, Birmingham, AL, U.S.A., CCCP from Aldrich, Milwaukee, WI, U.S.A. and cholesterol, nigericin, monensin and valinomycin from Sigma, St. Louis, MO, U.S.A. All other chemicals were of reagent grade.

Results

Pump- and hydrolytic activities of the Na^+/K^+-ATPase containing proteoliposomes

Proteoliposomes, reconstituted by the freeze-thaw/sonication procedure were able to extrude Rb^+ actively upon addition of ATP to the medium. At 22°C the reconstituted Na^+/K^+-ATPase pumped $^{86}Rb^+$ out of the preloaded vesicles with an initial velocity of 0.5 mmol/mg protein per h (based on total protein content). This velocity deviated from linearity after 30 s (not shown). Hydrolysis of ATP was assayed in the presence of ouabain (0.2 mM). P_i release was linear in time for about one min. Addition of the ionophores valinomycin + CCCP and nigericin increased the linearity of the P_i release (Fig. 1), whereas monensin did not change the rate of hydrolysis at all. The velocity of ATP hydrolysis by the proteoliposomes was about 10 to 20% of that of the purified enzyme (at 20°C). The incorporation of the enzyme was about 50% inside-out and 50% rightside-out (determined by opening the vesicles with detergent [13]. The non-incorporated part of the enzyme population was less than 10 percent on protein base as determined by separation of the proteoliposomes from the fragmented enzyme by sucrose gradient [14]. These molecules were inactive in hydrolysis and phosphorylation because of the presence of ouabain. The rightside-out oriented Na^+/K^+-ATPase molecules were also silent because of the presence of the ATP site intravesiculary, inaccessible to ATP, added to the medium. Only the inside-out oriented Na^+/K^+-ATPase molecules could be active in the different assays used: phosphorylation, ATP-hydrolysis and active transport.

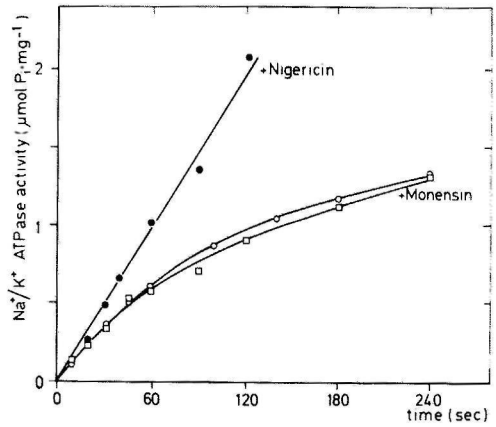

Fig. 1. ATP hydrolysis by reconstituted Na^+/K^+-ATPase. Na^+/K^+-ATPase reconstituted in liposomes (phosphatidylcholine 26, phosphatidylserine 2 and cholesterol 20 mg/ml) containing Tris (20 mM, pH 7.2), K^+ (10 mM) and Na^+ (100 mM) were preincubated with ouabain (1 mM; 60 min at room temperature). 10 μl of these proteoliposomes (protein concentration 1 mg/ml) were then incubated with 190 μl of the labeled ATP-containing medium (Mg^{2+}, 1 mM; K^+, 100 mM; Na^+, 10 mM; ATP, 1 mM and ouabain, 0.2 mM) at room temperature and the reaction was stopped with trichloroacetic acid at the indicated time (as described in Materials and Methods). Prior to incubation the proteoliposomes were preincubated with ionophores dissolved in ethanol (5 μl), to obtain a final concentration of 20 μM nigericin (●) or monensin (□). The control without ionophore (○) is also shown.

Ouabain sensitivity and phosphorylation level

The steady state level of phosphorylation of Na^+/K^+-ATPase containing membrane sheets was totally inhibited by ouabain ($I_{50} = 1$ μM), whereas only a partial decrease (< 25%) of the phosphorylation level of the Na^+/K^+-ATPase containing proteoliposomes was observed (Fig. 2). The decrease in phosphorylation level at low ouabain concentrations (< 10 μM) is due to the non-incorporated part of the enzyme. The resulting phosphorylation level at this ouabain concentration must be assigned to the inside-out oriented part of the pump molecules, which are not inhibited by ouabain, because they have their ouabain binding site intravesicularly. At higher ouabain concentrations a further decrease of the phosphorylation level could be seen, probably due to small amounts of ouabain diffusing into the proteoliposomes. In all further experiments the proteoliposomes were preincubated with 0.2 mM ouabain and 10 mM Mg^{2+} to make sure that the measured phosphorylation levels were due to inside-out oriented Na^+/K^+-ATPase molecules. The maximal level of phosphorylation for proteoliposomes containing 200 mM Tris with 100 mM Na^+, 5 mM Mg^{2+}, 15 μM ATP and 0.2 mM ouabain in the phosphorylation medium reached 30 to 40% (based on total protein content of the

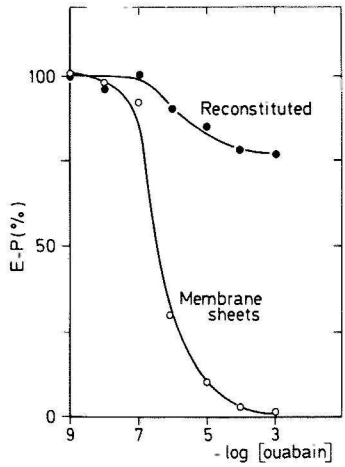

Fig. 2. Inhibition of reconstituted and fragmented Na^+/K^+-ATPase by ouabain. Proteoliposomes with the same lipid composition as described in Fig. 1, containing Tris (200 mM, pH 7.2) were preincubated with the indicated concentrations of ouabain in presence of Mg^{2+} (2.5 mM) at room temperature. After 60 min the phosphorylation reaction was carried out at the same temperature in a medium containing Na^+ (100 mM), Mg^{2+} (2.5 mM), Tris (100 mM; pH 7.0) and labeled ATP (1 μM) (●). Fragmented enzyme, preincubated with ouabain, was phosphorylated under the same conditions (○). In the absence of ouabain the steady-state phosphorylation level was 2.4 nmol/mg and 0.8 nmol/mg for the fragmented enzyme and the proteoliposomes, respectively.

proteoliposome suspension) of that of the purified enzyme under the same conditions, without ouabain.

Effects on the steady-state phosphorylation level by cytosolic and extracellular sodium

In proteoliposomes containing only intravesicular buffer solution (Tris 200 mM, pH 7.0) Na_{cyt} has a stimulating effect on the steady-state phosphorylation level. In a phosphorylation medium, containing 1 μM ATP and 5 mM Mg^{2+}, an enhancement of the phosphorylation level was according to the Michaelis-Menten formalism with a half-maximal stimulating concentration of 10.0 ± 2.5 mM (Fig. 3a). The $K_{0.5}$ values for Na^+ derived from this figure are represented in Table I. The $K_{0.5}$ value was increased by increasing K_{cyt} concentrations (Table II).

The Tris concentration at the cytoplasmic side had no effect on the steady-state phosphorylation level. In

TABLE I

$K_{0.5}$ Na_{cyt} values for reconstituted Na^+/K^+-ATPase

The $K_{0.5}$ values for Na^+ were derived from Fig. 3a after subtraction of the Na^+ insensitive level by Scatchard analysis.

ATP (μM)	$K_{0.5}$ Na_{cyt} (mM)
1.0	12
2.0	8.9
5.0	6.5
20.0	4.9

TABLE II

$K_{0.5}$ Na_{cyt} values for different K_{cyt} concentrations

Proteoliposomes, as described in the legend of Fig. 2, were phosphorylated in the presence of 5 mM Mg^{2+} and 1 μM ATP and K_{cyt} as indicated.

K_{cyt} (mM)	$K_{0.5}$ Na_{cyt} (mM)
0.0	10
0.1	12
1.0	20
10.0	23
200	100

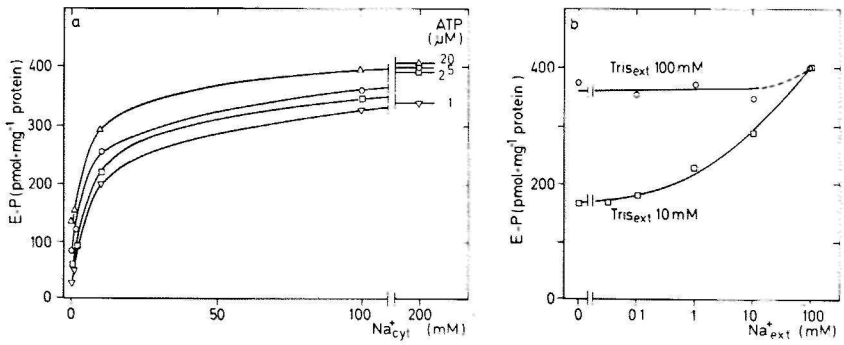

Fig. 3. Enhancement of the steady-state phosphorylation level by cytoplasmic and extracellular sodium. (a) Proteoliposomes, as described in Fig. 2 were phosphorylated in a medium containing Mg^{2+} (5 mM), Tris (20 mM, pH 7.0), choline chloride (to adjust osmolarity) and Na^+ (as indicated). Four different ATP concentrations were used for the phosphorylation: 1 μM (▽), 2 μM (□), 5 μM (○) and 20 μM (△). (b) The same phosphorylation experiment (ATP concentration 1 μM) was carried out with proteoliposomes with intravesicularly increasing Na^+ content and different Tris concentration: 10 mM (□) and 100 mM (○). Choline chloride was added intravesicularly in order to keep the osmolarity constant at 380 mosM. $K_{0.5}$ values were obtained after subtraction of the Na^+-independent level from Scatchard plots.

the absence of added Na_{cyt} the phosphorylation level was already 10 to 20% of the maximal level (with 200 mM Na_{cyt}). Increasing the ATP concentration from 1 to 20 μM increased this level to 35% of the maximum (Fig. 3a). The phenomenon, that ATP, Mg^{2+} and buffer were sufficient to give a (submaximal) level of phosphorylation indicates that Na^+ is not a unique cation to induce the phosphorylation. This level, which was reached in the absence of added Na^+, must be due to buffer stimulated phosphorylation as described by Schuurmans Stekhoven et al. [15,16]. Extracellular sodium increased the steady-state phosphorylation level in presence of 20 mM extracellular Tris, whereas with 100 mM extracellular Tris no further increase of the phosphorylation level could be observed (Fig. 3b). With 100 mM extracellular Tris the same phosphorylation level as with 100 mM Na_{ext} was obtained.

Cytoplasmic and extracellular Mg^{2+}

The role of Mg^{2+} in the reaction mechanism of Na^+/K^+-ATPase is rather complex [19]. Chelating agents have to be used in studies on the role of divalent cations in the reaction mechanism of Na^+/K^+-ATPase and have effects themselves, which cannot easily be explained [17,18]. In Na^+/K^+-ATPase-containing membrane sheets a maximal phosphorylation level could be reached in the presence of 50 mM Na^+ without adding any Mg^{2+} (Fig. 4). However, in the presence of 0.5 mM EDTA the steady-state phosphorylation level decreased and a dose-dependent increase in the phosphorylation level was found with a maximum between

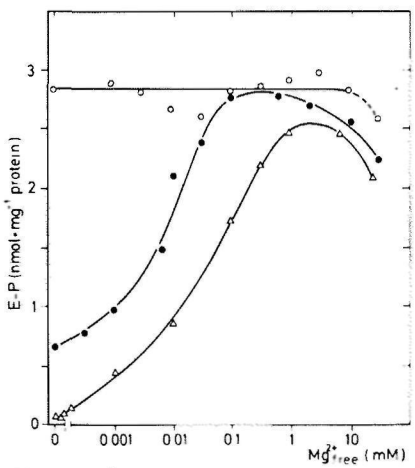

Fig. 4. Effect of Mg^{2+} and EDTA on the steady-state phosphorylation level of fragmented Na^+/K^+-ATPase. Purified enzyme was phosphorylated in a medium containing Na^+ (50 mM), Tris (70 mM, pH 7.0), choline chloride (30 mM), ATP (1 μM), EDTA and Mg^{2+} to get the indicated free Mg^{2+} concentration (0 mM (○), 0.5 mM (●) or 10 mM (△)).

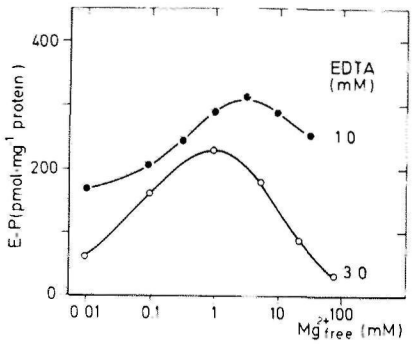

Fig. 5. Effect of cytoplasmic Mg^{2+} on the steady-state phosphorylation level in reconstituted Na^+/K^+-ATPase. Proteoliposomes as in Fig. 2, containing Tris (170 mM, pH 7.0) and choline chloride (30 mM) were preincubated with ouabain (0.2 mM) and Mg^{2+} (2 mM). After preincubation EDTA to a final concentration of 10 mM was added. Immediately thereafter the preincubated proteoliposomes were phosphorylated in a medium as described in the legend of Fig. 4. The EDTA concentration during the phosphorylation was diluted to 1 mM (●) and 3 mM (○), respectively.

0.1 and 1.0 mM Mg^{2+}. In the presence of 10 mM EDTA the steady-state phosphorylation level was nearly zero in the absence of added Mg^{2+} and the dose-response curve was shifted to the right. At higher Mg^{2+} concentrations there was a tendency to a lowering of the E-P level.

In the reconstituted system the Mg^{2+}-dependency can only be studied in the presence of EDTA, since Mg^{2+} is needed for the binding of ouabain to the non-incorporated and rightside-out oriented Na^+/K^+-ATPase. Fig. 5 shows that the phosphorylation level in the reconstituted system depended on the Mg_{cyt} concentration. An increase in the EDTA concentration lowered the E-P level at all Mg_{cyt} concentrations and shifted the dose-response curve to the left. The Mg_{cyt} concentration at which the maximal E-P level was reached was lower with higher EDTA concentrations too. In the reconstituted system the inhibitory effect at higher Mg^{2+} concentrations was much more pronounced than with the fragmented enzyme.

The Na_{cyt} dependency of the E-P level depended not only on the ATP concentration (Fig. 3a) but also on the Mg^{2+} concentration (Fig. 6). The E-P level at 1 mM Mg^{2+} was higher than at 5 mM. The effect of Mg^{2+}, however, was most pronounced in absence of Na_{cyt}. With 5 mM Mg^{2+} the steady-state phosphorylation level in absence of Na_{cyt} was less than 15% of the maximal level, whereas this was almost 50% with 1 mM Mg^{2+}.

The affinity for ATP was influenced by cytoplasmic Mg^{2+} as well as by cytoplasmic Na^+. An increase of the Mg^{2+} concentration increased the $K_{0.5}$ whereas increas-

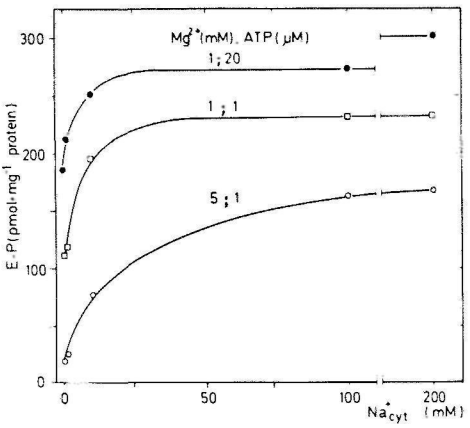

Fig. 6. Effect of cytoplasmic Mg^{2+} and ATP on the sodium dependency of the steady-state phosphorylation level. Proteoliposomes as described in Fig. 2 were phosphorylated in the presence of Tris (20 mM, pH 7.0), choline chloride (to maintain isoosmolarity) and either 5 mM Mg^{2+} and 2 μM ATP (○), 1 mM Mg^{2+} and 2 μM ATP (□), or 1 mM Mg^{2+} and 20 μM ATP (●).

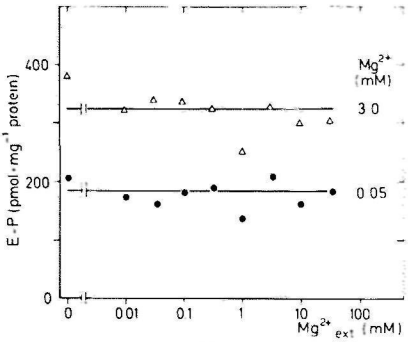

Fig. 7. Effect of extracellular Mg^{2+} on the steady-state phosphorylation level. Proteoliposomes (as in Fig. 2), loaded with Tris (170 mM, pH 7.0), choline chloride (to maintain isoosmolarity), EDTA (0.1 mM) and Mg^{2+} to obtain the indicated free Mg^{2+} concentrations were phosphorylated with two different cytoplasmic Mg^{2+} concentrations: 0.05 mM (●) and 3 mM (△).

ing the Na^+ concentrations decreased the $K_{0.5}$ (Table III).

Modification of the extracellular Mg^{2+} concentration had no effect on the steady-state phosphorylation level (Fig. 7). The variation in these experiments is due to the fact that for changes in the extracellular concentration the intravesicular composition has to be changed so that each point represents a separate proteoliposome preparation.

Cytoplasmic potassium

When the phosphorylation reaction was carried out with 1 μM ATP at 0 °C with at the extracellular side 150 mM Tris only a minor inhibitory effect of K_{cyt} on the steady-state phosphorylation level was observed at concentrations of K_{cyt} up to 10 mM (Fig. 8). At room temperature, however, K_{cyt} at low concentrations already considerably decreased the phosphorylation level.

TABLE III

ATP affinity for reconstituted Na^+/K^+-ATPase

Proteoliposomes, as described in the legend of Fig. 2, were phosphorylated in the presence of the indicated Mg^{2+} and Na^+ concentrations with increasing ATP concentrations. $K_{0.5}$ values were derived from Scatchard plots.

Na^+ (mM)	Mg^{2+} (mM)	$K_{0.5}$ ATP (μM)
0.2	1.2	1.25
1.0	5.0	1.54
20	5.0	0.77
20	1.2	0.40
100	5.0	0.24

Increase of the ATP concentration to 10 μM or replacement of the intravesicular Tris by Na^+ decreased this potassium sensitivity. Lowering the temperature to 15 °C under the latter conditions totally abolished the inhibitory effect of K^+.

An antagonism between Na_{cyt} and K_{cyt} on the steady-state phosphorylation level could be observed under conditions where the proteoliposomes were sensitive to low concentrations of K_{cyt}. At high Na_{cyt} concentrations (200 mM) the $K_{0.5}$ for K^+ was 0.6 mM whereas at low Na_{cyt} concentrations (10 mM) the $K_{0.5}$ was only 0.05 mM (Fig. 9).

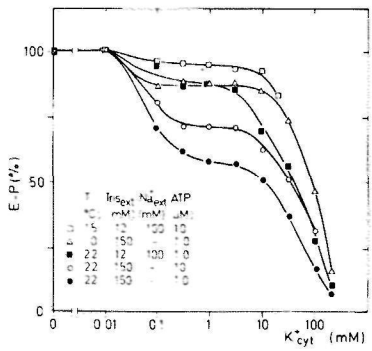

Fig. 8. Decrease of the steady-state phosphorylation level by cytoplasmic K^+. Proteoliposomes (phosphatidylcholine 20 mg/ml; cholesterol 6 mg/ml), containing Tris (150 mM, pH 7.0) were phosphorylated in the presence of Na^+ (20 mM), Mg^{2+} (5 mM), the indicated K_{cyt} concentrations and 1 μM ATP at 0 °C (○), at 22 °C (●) or at 22 °C with 10 μM ATP (○). Proteoliposomes with the same lipid composition, containing histidine (12 mM, pH 7.0) and Na^+ (100 mM) were phosphorylated with 1 μM ATP at 22 °C (■) or with 10 μM ATP at 15 °C (□).

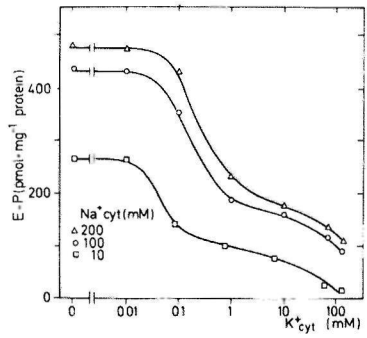

Fig. 9. Decrease of the phosphorylation level by cytosolic potassium at different cytosolic sodium concentrations. Proteoliposomes as described in the legend of Fig. 2 were phosphorylated in the presence of Mg^{2+} (5 mM), ATP (1 μM) and Na_{cyt} 10 mM (□), 100 mM (○) or 200 mM (△), with the K_{cyt} concentrations as indicated.

Under all conditions mentioned above a decrease in the steady-state phosphorylation level was observed at K_{cyt} concentrations above 10 mM. The I_{50} value for the latter effect was between 60 and 100 mM. This decrease is probably due to occupation of the Na^+ sites by K_{cyt} yielding an occluded E_2K conformation [20,21]. The decrease of the steady-state phosphorylation level was not due to an increase of the dephosphorylation rate, because the dephosphorylation rate in the presence of K_{cyt} was not significantly higher than in absence of this ligand (not shown).

Extracellular potassium

Fig. 10 shows, that K_{ext} reduced the steady-state phosphorylation level only for 15% at concentrations

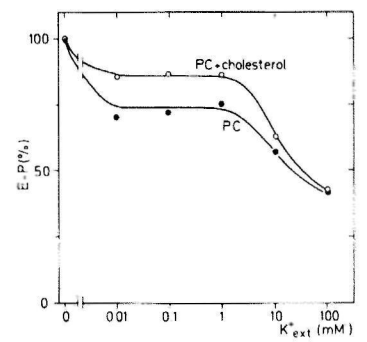

Fig. 10. Effect of extracellular K^+ on the steady-state phosphorylation level. Proteoliposomes with (○) and without (●) cholesterol (20 mg/ml, phosphatidylserine was 4 mg/ml and phosphatidylcholine 26 and 46 mg/ml, respectively) were prepared with different concentrations of extracellular K^+. The proteoliposomes contained additionally Tris (100 mM; pH 7.0) and choline chloride to maintain isoosmolarity. The phosphorylation medium was the same as in Fig. 2.

between 0.01 and 1 mM. At higher concentrations a further decrease in the E-P level was observed. The small decrease at low K_{ext} concentrations is unexpected since in fragmented enzyme preparations a nearly completely decrease in E-P is found. An explanation could be that in the reconstituted system the amount of E_2P is very small. Yoda and Yoda [22,23] have suggeted that part of the phosphorylated intermediate is in the E*P form (already proposed by Nørby and Klodos [28,29]) which only can be deposphorylated by K_{ext} after conversion into E_2P. The ratio of E_2P to $E^*P + E_1P$ has reported to be increased with decreasing cholesterol content of Na^+/K^+-ATPase-containing liposomes. The above experiment was therefore repeated with proteoliposomes containing no cholesterol, whereas in all other experiments the cholesterol content was 40%. At K_{ext} concentrations between 0.01 and 1 mM there was a significant further decrease in the steady-state phosphorylation level compared to that in cholesterol containing proteoliposomes. At K_{ext} concentrations above 10 mM the E-P level further decreased as was the case for the cholesterol containing proteoliposomes. An explanation for the latter inhibition could be the formation of an E_2K conformation which cannot be phosphorylated anymore.

Dephosphorylation

Since steady-state phosphorylation levels are the result of phosphorylation and dephosphorylation reactions, interpretation of the effects of added K^+ on the E-P level is complex. Therefore the phosphorylated intermediate was first prepared during 10 s, whereafter the radioactive ATP was diluted 10000-fold. The E-P level was measured at various time points. Dephosphorylation curves showed non-linear behaviour in a semilogarithmic plot. Fig. 11 indicates that the dephosphorylation rate in the first 3 s was faster than in the following 6 s. The steady-state phosphorylation level in proteoliposomes containing a high content of cholesterol (Fig. 11a) was only 5% reduced when 10 mM K_{ext} was present in the vesicles either at 37 °C or at 0 °C. At 37 °C the steady-state phosphorylation level was about half of that at 0 °C. The dephosphorylation rate in the first 3 s was, however, faster in vesicles containing K_{ext} than in vesicles without K_{ext}. The relative increase of the dephosphorylation rate was larger at 0 °C than at 37 °C.

The proteoliposomes without cholesterol (Fig. 11b) showed a larger difference in the steady-state phosphorylation level at 0 °C between vesicles with and without K_{ext} (34%). The fast dephosphorylation rate in the first 3 s was for both preparations the same.

Addition of 1 mM ADP also increased the rate of dephosphorylation suggesting that part of the phosphorylated intermediate was ADP-sensitive. When the phosphointermediate was formed in the presence of

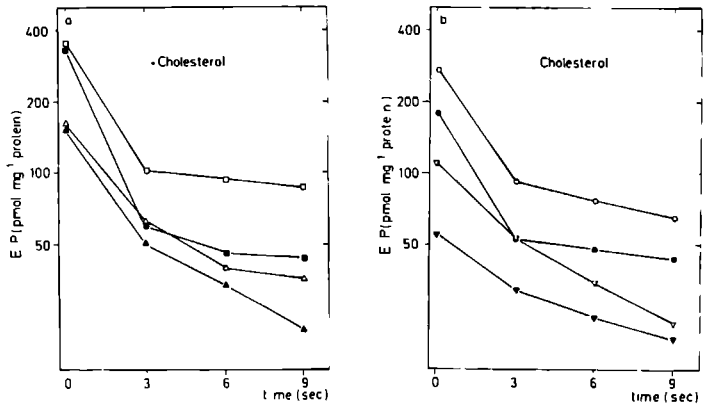

Fig 11 Dephosphorylation of reconstituted Na^+/K^+ ATPase (a) Proteoliposomes (as in Fig 8) containing Tris (12 mM pH 7 0) Na^+ (50 mM) and choline chloride (100 mM) (open symbols) or Tris (12 mM pH 7 0) Na^+ (50 mM) choline chloride (90 mM) and K^+ (10 mM) (closed symbols) were phosphorylated during 10 s and dephosphorylated during the indicated time Reactions were carried out at 0 °C (squares) and 37 °C (triangles) Proteoliposomes containing no Na^+ showed an identical dephosphorylation pattern (b) The same dephosphorylation experiment was carried out with proteoliposomes without cholesterol (phosphatidylcholine was 26 mg/ml) loaded with Tris (200 mM pH 7 0) (open symbols) or Tris (190 mM pH 7 0) and K^+ (10 mM) (closed symbols) Dephosphorylation at 0 °C is represented by circles and at 37 °C by triangles

K_{cyt}, the ADP-sensitive fraction was reduced to very low levels (not shown)

Discussion

Proteoliposomes as tools for sidedness studies

Na^+/K^+-ATPase reconstituted in lipid vesicles provides a good system to study such properties as transport and sidedness of ligand activation or inhibition on transport and overall or partial reactions The composition of the intravesicular medium is totally under control and can be chosen during preparation of the proteoliposomes Because it is possible to separate (or exchange) the extravesicular medium by gel filtration or ion exchange chromatography for any medium of choice, control of the extravesicular medium is also guaranteed In studying a fast process as phosphorylation, where only short periods of incubation with ligands are necessary, the hazard of leakage of ligands in either direction is almost excluded In the present study proteoliposomes were always preincubated with ouabain and Mg^{2+} at a concentration which inhibits more than 97% of non-incorporated ATPase molecules The rightside-out oriented Na^+/K^+-ATPase has its ATP binding site inside the vesicle, which cannot be reached by externally added ATP This leaves only a single population the inside-out incorporated pump molecules as subject for our phosphorylation studies The orientation of the latter pump molecules makes the cytoplasmic side accessible from the medium hence variation in the composition of the extravesicular medium represents a variation at the cytoplasmic side of these ATPase molecules

Na^+ and buffer cations

Titrating the Na^+ concentration in the phosphorylation medium (Na_{cyt}), which also contained Mg^{2+} and ATP, increased the phosphorylation level in a similar way as with fragmented Na^+/K^+-ATPase The mechanism of this activation is probably the formation of the E_1 conformational state of the enzyme (Fig 12), which can bind ATP and subsequently can be phosphorylated [24] The maximal level of phosphorylation, however, was only attained when the extracellular (intravesicular) medium contained a minimal concentration of Tris The latter buffer could be successfully replaced by sodium or by other buffers like imidazole, triethanolamine, triallylamine or histidine, but not by choline chloride or sorbitol This indicates that the presence of either sodium or one fo the amine buffers at the extracellular side is a prerequisite for high phosphorylation levels It im-

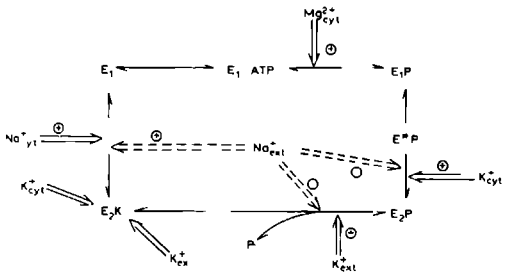

Fig 12 Modified Post Albers Scheme for Na^+/K^+ ATPase in which the sidedness of ligands is given (+) indicates a stimulatory and (−) an inhibitory effect on the transitions (in a clockwise direction of the reaction cycle) = = = > indicate the three possible sites of effects in the reaction cycle as discussed in the text

plicitly proves the presence of extracellular binding sites for Na^+ and amine buffers The mechanism of this extracellular effect of Na^+ or amine buffers is not clear but could be due to inhibition of the dephosphorylation rate (Fig 12) as described by Schuurmans and Stekhoven et al [25] and by Fukushima [26] Retardation of the dephosphorylation reaction would lead to accumulation of phosphorylated intermediates An alternative explanation is that the enzyme is partly in the E_2 conformation and that extracellular ligands induce an E_1 conformation, which can bind ATP and can subsequently be phosphorylated (Fig 12) Such allosteric effect of Na^+ has been reported by Karlish and Stein [27] A third explanation is that the extracellular ligands induce a shift to the left in the $E_1P \leftrightarrow E_2P$ equilibrium (Fig 12) Such a shift would in itself not have an effect on the phosphorylation level, but if the hydrolysis of E_2P during the three seconds of the phosphorylation period is faster than that of E_1P it would lead to an increase in the steady-state phosphorylation level [33] Further study is required to establish which of these three alternatives is valid

The effects of K^+

Extracellular K^+ is supposed to reduce the steady-state phosphorylation level by increasing the dephosphorylation rate (Fig 12) However, under the standard conditions mentioned above K_{ext} reduces the steady-state phosphorylation level in proteoliposomes containing high cholesterol by only 15% According to the third explanation mentioned above the reduction of the phosphorylation level by K_{ext} occurs through the E_2P part of the phosphorylated intermediates This suggests that in the proteoliposomes containing high levels of cholesterol most of the phosphorylated intermediates were either in the E_1P or the E^*P form The latter form is postulated by Nørby and Klodos [28 29] In proteoliposomes containing no cholesterol low concentrations of K_{ext} were able to lower the steady-state phosphorylation level by 30% indicating that the amount of E_2P was increased This finding is in agreement with the observation of Yoda and Yoda [23] that cholesterol shifts the $E^*P \leftrightarrow E_2P$ equilibrium to the left The reduction of the steady-state phosphorylation level at high concentrations of K_{ext} is consistent with results of Karlish and Stein [31] Blostein and Chu [32] however, found a total reduction by 100 μM K_{ext} of the steady-state phosphorylation level in erythrocyte ghosts without Na_{ext} and no reduction in ghosts with Na_{ext} This might indicate, that our reconstituted system with high intravesicular buffer concentrations represents a system with properties in between the two extremes of the ghost preparations with and without Na_{ext} Possibly the enzyme is mainly in an E^*P form and only a minor part in the E_2P form A similar suggestion was made by Yoda and Yoda [33]

Both in cholesterol-containing and in cholesterol-free proteoliposomes K^+ concentrations above 1 mM gave a further decrease in the steady-state phosphorylation level The high K^+ concentration needed for this effect makes it unlikely that the effect is due to an increase in the dephosphorylation rate We suggest that an E_2K complex was formed which could not be phosphorylated anymore (Fig 12)

Reduction of the steady-state phosphorylation level by low K_{ext} concentrations could only be observed under special conditions A high-affinity K^+ binding site is proposed by other authors [27,34 35] Presence of 150 mM Tris, absence of Na_{ext}, low ATP concentration and an incubation temperature of 22 °C resulted in a 40–50% reduction in the steady-state phosphorylation level at 1 mM K_{ext} Increase in the ATP or Na_{ext} concentration (or a decrease in the Tris concentration) and a lowering of the temperature led to a diminishment of this effect This might be the reason, why others [23 32] observed that the phosphorylation in inside-out oriented Na^+/K^+-ATPase in proteoliposomes and erythrocytes was insensitive to K_{ext} Under the given conditions (high extracellular buffer concentration and 22 °C the dephosphorylation rate of E_2P is considered to be high, but at the same time the intravesicular buffer blocks the transformational change from E^*P to E_2P [30] These conditions make it possible to obtain a shift of E^*P to E_2P The blockade of this conformational change by extracellular Tris was apparently overruled by K_{ext} (Fig 12) and a decrease in the phosphorylation level was observed as a consequence of the fast dephosphorylation With low extracellular Tris or histidine concentrations the E^*P to E_2P conformational change was apparently not inhibited but the dephosphorylation reaction was slow As a consequence the change of E^*P to E_2P could not be observed as a decrease in the steady-state phosphorylation level At 0 °C K_{ext} apparently was not able to induce the shift between E^*P and E_2P and therefore no decrease in the steady-state phosphorylation level could be observed The latter result is in accordance with that of Blostein and Chu [32]

At higher K_{ext} concentrations (10–200 mM) a decrease of the phosphorylation level was observed under all conditions mentioned above, as was the case with high K_{ext} concentrations This reduction of the level can be explained by occupation of the low-affinity K sites Occupation of these sites by K^+ prevents the conversion of E_2 to E_1 which means that part of the enzyme cannot be phosphorylated anymore [31] This is confirmed by the antagonism between Na_{ext} and ATP on the one and K_{ext} on the other hand The increase by K^+ of the dephosphorylation rate of fragmented enzyme is in the light of our model the result of two simultaneous effects on the enzyme (1) the shift of E_1P and E^*P towards E_2P induced by K_{ext} (which is probably very fast) and (2) the increase of the dephosphorylation rate of E_2P by

K_{ext}. A remarkable discrepancy between membrane sheets and reconstituted enzyme was found. In presence of 10 mM K^+ and 100 mM Na^+ the steady-state phosphorylation level of Na^+/K^+-ATPase in fragmented membrane sheets was practically zero [15] With 10 mM K^+ and 100 mM Na^+ at both sides of the reconstituted enzyme we found phosphorylation levels which reached 26% of the maximum in absence of K^+ under otherwise the same conditions The results obtained with the reconstituted enzyme, however, establish the presence of K^+-binding sites both at the extracellular and at the intracellular side of the membrane

Effects of Mg^{2+}

Variation of the free extracellular Mg^{2+} concentration has no influence on the steady-state phosphorylation level The cytoplasmic Mg^{2+} concentration however increases the steady-state phosphorylation level at low Mg^{2+} concentrations and lowers it at high Mg^{2+} concentrations The magnitude of the effect depends similar to that in the fragmented enzyme on the EDTA concentration This effect of EDTA, which can be observed even more clearly in fragmented enzyme [17,28], is not easy to explain Possible explanations are direct inhibitory effects of EDTA, complexation of unknown divalent cations which function can be imitated by Mg^{2+} or a change in the lipid–protein interaction which can only be reversed by high Mg^{2+} concentrations. In the reconstituted enzyme the inhibitory effect of high Mg^{2+} concentrations is more pronounced than in the fragmented enzyme In the proteoliposomes Mg^{2+} stimulates the phosphorylation in the presence of Na_{cyt} and low ATP concentrations In the absence of Na_{cyt} however high levels of phosphorylation can only be obtained when the Mg^{2+} concentration is below 1 mM Higher concentrations of Mg^{2+} strongly decrease the phosphorylation level This antagonism has also been observed for the buffer stimulated phosphorylation of fragmented enzyme [15] In the absence of Na_{cyt} and at relatively high ATP concentration (20 μM) the reconstituted enzyme is phosphorylated to 80% of the maximal level attained in presence of saturating Na_{cyt} This level is obtained with extracellular imidazole, but also with (high concentrations of) Tris With fragmented enzyme, where ligands can approach the enzyme from both sides, Tris does not stimulate phosphorylation in the absence of Na^+ This different behaviour of Tris can be explained by its inhibitory capacity at the cytoplasmic side, or by the different lipid environment of the reconstituted enzyme The stimulating effect of buffers is only observed at the extracellular side, whereas Na^+ has a dual effect by enhancing phosphorylation from the cytoplasmic as well as from the extracellular side

Conclusions

In conclusion our findings indicate that· (1) High buffer (or Na^+) concentrations are necessary at the extracellular side to obtain maximal phosphorylation levels. (2) Amine buffer-stimulated phosphorylation is suppressed by Mg^{2+} and low ATP concentration (3) Cytoplasmic K^+ reduces the steady-state phosphorylation level at low concentrations by shifting the EP intermediates towards the E_2P conformation and at high concentrations via occupation of the low-affinity K^+-sites by yielding the K^+-occluded state of the enzyme (4) Extracellular K^+ reduces the steady-state phosphorylation level partially, i.e only those EP intermediates which are in the E_2P conformation will be hydrolysed (5) Binding sites are demonstrated for: K_{cyt} (high and low affinity), K_{ext} (high and low affinity), Na_{cyt}, Na_{ext}, Mg^{2+} (cytoplasmic) and amine buffers at the extracellular side

Acknowledgements

The authors would like to thank Dr. F.M A H Schuurmans Stekhoven for his useful comments on this manuscript. This investigation was financially supported by the Netherlands Organization for Scientific Research (N W.O), through the Netherlands Biophysics Foundation

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Sidedness of the effect of amines on the steady-state phosphorylation level of reconstituted (Na^++K^+)-ATPase
ATP-ase.

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Biochim. Biophys. Acta 987 (1989) 75-82

Biochimica et Biophysica Acta 987 (1989) 75–82
Elsevier

BBAMEM 74669

Sidedness of the effect of amines on the steady-state phosphorylation level of reconstituted Na^+/K^+-ATPase

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(Received 11 July 1989)

Key words ATPase, Na^+/K^+-, Phosphorylation, Amine effect, Cytoplasmic side, Extracellular side

The sidedness of the effects of several amines on the steady-state phosphorylation level of rabbit kidney Na^+/K^+-ATPase has been studied with the enzyme incorporated in phosphatidylcholine-cholesterol containing proteoliposomes. The presence of ouabain prevented phosphorylation of non-incorporated or rightside-out incorporated enzyme, so that only the inside-out incorporated Na^+/K^+-ATPase molecules were studied. Addition of either Na^+ or several amines to the extracellular side of the enzyme led to an enhancement of the steady-state phosphorylation level obtained with optimal concentrations of Na^+, Mg^{2+} and ATP at the cytosolic side. The series imidazole > Na^+ > triallylamine > Tris > ethylenediamine showed a decrease in affinity. Histidine, sorbitol and choline chloride had no effect at the extracellular side. This means that in addition to the well-known cytosolic ligands either Na^+ or a positively charged amine buffer has to be present extracellularly in order to obtain an optimal phosphorylation level. At the cytoplasmic side the tested amines exerted different effects. (i) Imidazole and triallylamine enhanced the steady-state phosphorylation level when the extracellular conditions were optimal (saturating amine concentration). (ii) Tris and ethylenediamine decreased the steady-state phosphorylation level and (iii) histidine had no effect. The cytoplasmic effects of the amine compounds correlate with those described by Schuurmans Stekhoven et al. (Biochim. Biophys. Acta 937 (1988) 161–171) for the unsided preparation. The extracellular effects, however, are apparently masked in experiments with fragmented enzyme preparations and are assumed to be potentiating effects which make the enzyme ready for phosphorylation upon a cytoplasmic trigger (e.g. Na^+).

Introduction

Na^+/K^+-ATPase as a member of the 'P'-type ATPase [1] forms a covalent phosphorylated intermediate as part of its reaction cycle The phosphorylation reaction has extensively been studied [2] It has become clear that the presence of Mg^{2+} and Na^+ [3,4] besides the substrate is a prerequisite for this reaction. In a recent study [5], however, the enhancement of the phosphorylation level by imidazole in the absence of Na^+ was reported. From this report it was clear that imidazole was able to substitute for Na^+ in the phosphorylation reaction of the Na^+/K^+-ATPase, but the mechanism of this phenomenon remained obscure. In a further study [6] some other amine compounds were found to enhance the phosphorylation reaction, whereas others were without effect or even inhibitory versus Na^+ or other stimulating compounds. This made the picture even more complex

The assignment of the sites of action of those compounds could give some clues about a possible mechanism. Since the interaction of those charged compounds is probably mainly electrostatic it is difficult to derive information from binding studies So far binding studies were not conclusive. An other approach to obtain more insight in this process is to study the sidedness of action of the amine compounds

In a recent paper [7] we presented a study on the sided effects of physiologically important ions with respect to the phosphorylation reaction in reconstituted Na^+/K^+-ATPase With this preparation it appeared to be possible to obtain information about the sidedness of

Abbreviations Tris, tris(hydroxymethyl)aminomethane, EDTA, ethylenedinitrotetraacetic acid, K_{cyt}, Na_{cyt} and Mg_{cyt} represent K^+, Na^+ and Mg^{2+} concentrations at the cytosolic side, respectively K_{ext}, Na_{ext} and Mg_{ext} represent these concentrations at the extracellular side

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action of the amine compounds With this sided preparation the cytoplasmic and extracellular effects could be separated By this separation stimulatory and inhibitory effects turned out to be located on different sides of the membrane and could be studied separately

A model for the mechanism of action of the amine compounds is proposed This model throws some light on observations in unsided preparations which are difficult to interpret

Materials and Methods

Preparation of Na^+/K^+-ATPase

Na^+/K^+-ATPase from rabbit kidney outer medulla has been prepared according to the method described by Jørgensen [8] About 340 mg microsomes (on protein base) were incubated for one hour at 20 °C in a medium containing 0 58 mg/ml sodium dodecyl sulfate and 25 mM imidazole-HCl (pH 7 4), 3 mM ATP and 2 mM EDTA (final protein concentration 1 45 mg protein per ml) After the extraction the microsomal suspension was centrifuged on a sucrose gradient (0 50%) The ATP of the pooled fractions of the gradient was removed by incubation at 37 °C in presence of Na^+, Mg^{2+} and K^+ and subsequent washing The obtained membrane fragments, enriched in Na^+/K^+-ATPase, were stored in imidazole buffer (25 mM, pH 7 4) containing 10% sucrose The specific Na^+/K^+-ATPase activity of the preparations ranged from 1 0 to 1 6 mmol P_i formed per mg protein per hour

Preparation of liposomes

Liposomes have been prepared by a reversed phase evaporation method as described by Szoka and Papahadjopoulos [9] Mixtures of cholesterol, phosphatidylcholine and phosphatidylserine (ratio indicated in text and legends) in chloroform were evaporated under a stream of nitrogen to remove the organic solvent After repeated washing with diethylether, a 1 1 mixture of diethylether and buffer solution of different composition was added and the solution thoroughly mixed on a Vortex mixer, while the diethyl ether was again slowly evaporated by a stream of nitrogen The final lipid content was between 20 and 50 mg/ml After all ether had disappeared the liposomes formed were sonicated for 30 min in a Branson sonicator bath at maximal output

Reconstitution procedure

Purified Na^+/K^+-ATPase (5 mg/ml) in 20 mM imidazole buffer (pH 7 2) was partially solubilized by incubation with cholate (final concentration 0 91% w/v) during 1 min at room temperature This mixture was then added to a 10-fold volume of liposomes, giving a lipid to protein ratio of 40 to 100 (on weight basis) After thorough mixing, the preparation was frozen in liquid nitrogen or in a mixture of dry ice and acetone and subsequently thawed at room temperature This freezing and thawing procedure was repeated twice Thereafter the vesicle suspension was sonicated for 6 min in a Branson sonicator bath (maximal output) Detergent was removed from the proteoliposomes by centrifuging aliquots of the suspension over a 10-fold volume Sephadex G-25 (coarse) column (equilibrated with the appropriate buffer solution in a syringe) This centrifugation step lasted 5 min ($100 \times g$) and was repeated once More than 99 9% of the cholate was removed by this procedure [10]

ATP hydrolysis

The ATP hydrolysis was determined as the release of $^{32}P_i$ from [γ-^{32}P]ATP [11] To 10 μl proteoliposomes, containing compounds as indicated in the legends, 190 μl medium containing ouabain (0 2 mM), 1 mM Mg^{2+} and 1 0 μM labeled ATP were added at 20 °C and incubated for 10–30 s For blank values the reconstituted Na^+/K^+-ATPase was denatured with trichloroacetic acid prior to incubation with the hydrolysis medium The $^{32}P_i$ production was measured after stopping the reaction at a given time through addition of 0 4 ml 10% trichloroacetic acid followed by mixing with 0 4 ml 20% (w/v) aqueous charcoal suspension The charcoal adsorbs the adenosine phosphates from the medium, but leaves P_i in solution The suspension was mixed thoroughly repeatedly (three times) for 10 s every 5 min Thereafter the charcoal was sedimented by centrifugation for 10 min at $2000 \times g$ at 0 °C Aliquots (0 2–0 5 ml) were taken from the supernatant, mixed with 4 5 ml liquid scintillation fluid (Aqualuma Plus) Radioactivity was measured with a liquid scintillation counter

Phosphorylation

Phosphorylation of the reconstituted Na^+/K^+-ATPase was carried out at 22 °C at pH 7 0 The ATP concentrations varied between 0 2 and 20 μM (The Radiochemical Centre, Amersham, U K , specific radioactivity 3000 Ci/mol) The reaction was started by rapid mixing of 10 μl proteoliposomes (preincubated with 0 2 mM ouabain and 10 mM Mg^{2+}) with 90 μl of the medium containing ATP and the other ligands The reaction was stopped after 3 s by addition of 3 ml 5% (w/v)trichloroacetic acid, containing 100 mM phosphoric acid The denatured phosphoprotein was filtered on a 1 2 μm pore width Selectron filter (Schleicher and Schull, Dassel, F R G), which was then washed three times with 3 ml of the stopping solution Incorporated ^{32}P was determined by liquid scintillation counting For blank values the proteoliposomes were mixed with the stopping solution prior to addition of the ATP solution

Dephosphorylation

After phosphorylation during 10 s at room temperature 900 μl of the dephosphorylation medium was ad-

ded to the phosphorylation mixture (100 μl). The dephosphorylation mixture contained, apart from the buffer and cations, 1 mM unlabeled ATP in order to dilute the labeled ATP 1000-fold. Together with the 10-fold dilution of the volume of the medium the final dilution of the labeled ATP was 10000 in order to avoid further phosphorylation by the labeled ATP. After rapid mixing the dephosphorylation reaction was stopped (at the time indicated) by addition of 5 ml 5% (w/v) trichloroacetic acid containing 100 mM phosphoric acid. After stopping the reaction, the mixture was further treated as described in the phosphorylation procedure.

Materials

ATP and Tris were purchased from Boehringer, Mannheim, F.R.G. [γ-^{32}P]ATP and ^{86}Rb were obtained from Amersham, Buckinghamshire, U.K.; phosphatidylcholine (egg) and phosphatidylserine (bovine brain) were purchased from Avanti Polar Lipids, Birmingham, AL, U.S.A., and cholesterol, from Sigma, St. Louis, MO, U.S.A. All other chemicals were of reagent grade.

Results

Effect of extracellular amine buffers and Na^+ on the steady-state phosphorylation level of Na^+/K^+-ATPase containing proteoliposomes

Proteoliposomes, loaded with 100 mM Na^+, 10 mM Tris (pH 7.0) and 82 mM sorbitol, exhibited a maximal phosphorylation level under optimal cytoplasmic phosphorylation conditions (see below). This level could not be further increased by any change in the extracellular (intravesicular) composition, but could be decreased by lowering the extracellular (intravesicular) Na^+ concentration. Replacing the extracellular Na^+ by sorbitol under the above mentioned phosphorylation conditions at the cytoplasmic (extravesicular) side resulted in a 55% reduction of the steady-state phosphorylation level (Fig. 1). The enhancement of the phosphorylation level by intravesicular Na^+ was half maximal at about 1 mM. A further (25%) decrease of the steady-state phosphorylation level occurred when the 10 M intravesicular Tris was also replaced by sorbitol. In the absence of extracellular (intravesicular) Na^+ and Tris and in the presence of 300 mM intravesicular sorbitol only 20–30% of the above mentioned optimal phosphorylation level was obtained.

Increasing the intravesicular Tris concentration up to 100 mM stimulated the phosphorylation to the same level as obtained with intravesicular Na^+. The $K_{0.5}$ for Tris was about 6 mM (Fig. 1). Fig. 1 also shows that the effect of the extracellular ligands was not due to a change in ionic strength of the intravesicular medium. Increasing the ionic strength of the intravesicular medium by replacing extracellular sorbitol by choline chloride did not increase the steady-state phosphorylation level above the level with extracellular sorbitol alone.

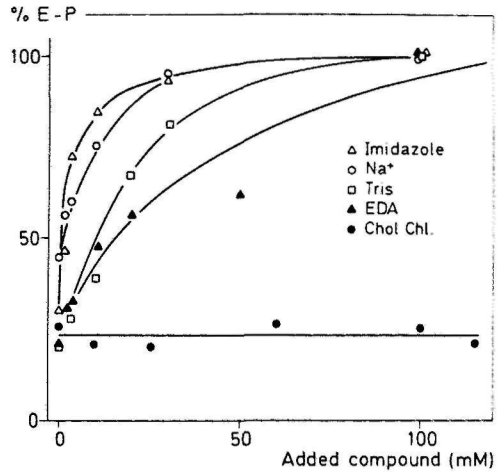

Fig. 1. Enhancement of the steady-state phosphorylation level by extracellular compounds. Na^+/K^+-ATPase reconstituted in liposomes (phosphatidylcholine 26, phosphatidylserine 2 and cholesterol 20 mg/ml) containing the compounds in concentrations as indicated in the figure (pH 7.2) were preincubated with ouabain (0.2 mM; 60 min at room temperature) in the presence of 10 mM Mg^{2+} and 150 mM Tris (pH 7.2). Sorbitol was added intravesicularly to maintain osmolarity. The proteoliposomes (protein content 0.5 mg/ml) were then phosphorylated with 1.0 μM labeled ATP in the presence of 100 mM Na^+, 3 mM Mg^{2+} and 51 mM Tris (pH 7.0). Further details of the phosphorylation reaction are described in Materials and Methods. The $K_{0.5}$ values for imidazole (△), Na^+ (○), Tris (□), ethylenediamine (EDA) (▲) and choline chloride (●) were obtained by Lineweaver-Burk and Scatchard analysis and given in the text. In the series with extracellular Na^+ 10 mM extracellular Tris was also present.

Other buffers tested exhibited a similar trans-effect on the steady-state phosphorylation level as Tris and Na^+. Imidazole and ethylenediamine (EDA) increased the phosphorylation level from the extracellular side with $K_{0.5}$ values of about 1 and 5 mM, respectively (Fig. 1). Triallylamine (TAA) also increased the phosphorylation level from the extracellular side, but unlike the other buffers tested above not according to the Michaelis-Menten formalism. After enhancement of the phosphorylation level by intravesicular triallylamine with a half maximal effect of 2–3 mM, a further increase of the intravesicular triallylamine concentration resulted in a considerable decrease of the phosphorylation level (Fig. 2), to be compared with the secondary inactivation also encountered in the open membrane system [12]. The maximal level of all the buffers tested however was the same as the maximal level obtained with intravesicular Na^+. The only buffer tested so far, which failed to increase the phosphorylation level from the extracellular side, was histidine (Fig. 2). Increasing the intravesicular histidine concentration up to 50 mM

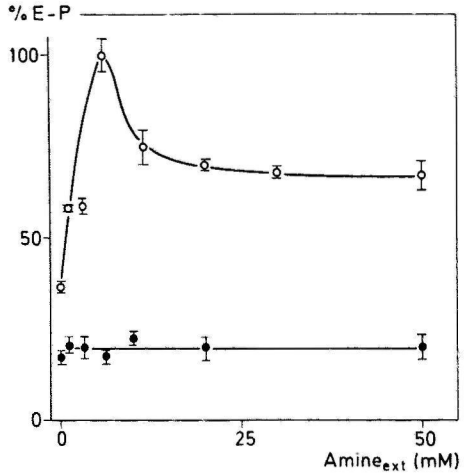

Fig. 2. The effect extracellular triallylamine (TAA) and histidine on the steady-state phosphorylation level. Proteoliposomes with the same lipid composition as described in Fig. 1, loaded with triallylamine (TAA) and histidine in the indicated concentrations were phosphorylated under the same conditions as described in Fig. 1. In the proteoliposomes containing triallylamine (○) 10 mM Tris (pH 7.0) was also present. The phosphorylation level of the proteoliposomes containing histidine are presented by (●). The symbols represent the mean ± S.D. of three experiments carried out in duplicate.

did not alter the steady-state phosphorylation level above the 20% obtained in the absence of intravesicular buffers.

The dephosphorylation rate of the phosphorylated intermediate in the presence of a low (10 mM) extracellular Tris concentration was not significantly different from that in the presence of a high (180 mM) extracellular Tris concentration: 0.25 ± 0.06 and 0.27 ± 0.07 s^{-1}, respectively. Furthermore the Na^+-dependent ATPase activity was linearly related to the phosphorylation level, independent from the type of amine or Na^+ extracellularly present (Fig. 3). This made the slow (1.66 ± 0.19 s^{-1}, at 20 °C) turnover of the enzyme (P_i production per phosphorylated intermediate) independent of the phosphorylation level.

Effects of cytoplasmic (extravesicular) amine buffers on the steady-state phosphorylation level

When Na^+/K^+-ATPase, reconstituted in lipid vesicles loaded with 165 mM Tris (pH 7.0), was phosphorylated with labeled ATP the obtained steady-state phosphorylation level increased with the cytoplasmic Na^+ concentration [7]. The phosphorylation level in the presence of cytoplasmic Tris and in the absence of added Na^+ depended on the cytoplasmic ATP and Mg^{2+} concentrations [7]. At low free Mg^{2+} concentrations the level was high and diminished above 3 mM Mg^{2+}. In the presence of high (5 mM) Mg^{2+} and low (1

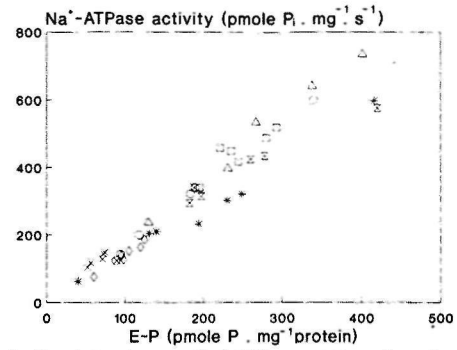

Fig. 3. Correlation between Na^+-ATPase activity and steady-state phosphorylation level. Protoliposomes as described in Fig. 1 containing Na^+ (△), Tris (○), imidazole (⧖), triallylamine (□), ethylenediamine (*), histidine (×) and choline chloride (◇) in the concentration ranges as shown in Figs. 1 and 2 were phosphorylated as described in Fig. 1 and Na^+-stimulated ATP hydrolysis was determined as described in Materials and Methods.

μM) ATP and in the absence of Na_{cyt} no influence on the phosphorylation level by variation of cytoplasmic Tris was observed (sorbitol being used to maintain isoosmolarity). Under these conditions the obtained phosphorylation level was low (only 2% of the maximal level with 100 mM Na_{cyt}). Imidazole and triallylamine in contrast to Tris markedly enhanced the steady-state phosphorylation level to 11 and 45% of the maximal

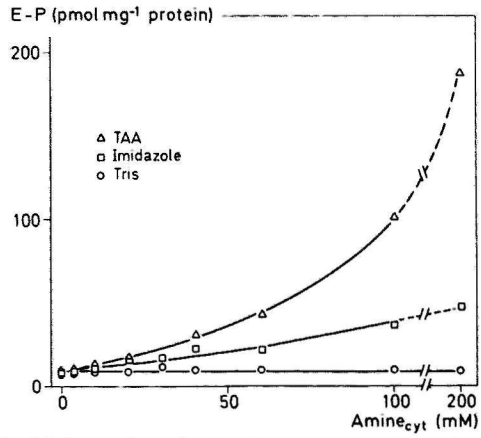

Fig. 4. Influence of cytoplasmic amine compounds on the steady-state phosphorylation level. Proteoliposomes as described in the legend of Fig. 1 loaded with 200 mM Tris (pH 7.0) were phosphorylated as described in Materials and Methods in the absence of Na_{cyt} and in the presence 1 μM ATP, 5 mM Mg^{2+} and the amino compounds as indicated; triallylamine (△), imidazole (□) and Tris (○). The phosphorylation level obtained with saturating Na_{cyt} was 610 pmol/mg protein. Sorbitol was added to the extravesicular medium to maintain osmolarity.

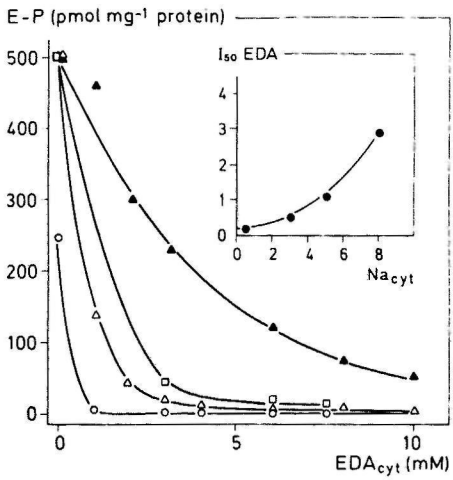

Fig. 5. Decrease of the steady-state phosphorylation level by cytoplasmic ethylenediamine (EDA) in the presence of different Na_{cyt} concentrations. Proteoliposomes as in Fig. 3 were phosphorylated as described in Fig. 3. The cytoplasmic medium contained ethylenediamine in the indicated concentrations and 0.5 (○), 3 (△), 5 (□) and 8 (▲) mM Na^+. The inset shows the dependency of the I_{50} value of ethylenediamine on the Na_{cyt} concentration.

level obtained with 100 mM cytoplasmic Na^+, respectively (Fig. 4). As with the effects of the amine buffers at the extracellular side, the influence on the phosphorylation level of these ligands at the cytoplasmic side was not due to ionic strength effects, as proven by choline chloride. Increasing the ionic strength along with the choline chloride concentration at the cytoplasmic side had no effect on the steady-state phosphorylation level (sorbitol being used to maintain isoosmolarity). Moreover histidine was also without effect on the steady-state phosphorylation level when added at the cytoplasmic side under the same conditions as choline chloride and the other buffers (not shown).

Ethylenediamine at the cytoplasmic side, in contrast to the former ligands, strongly reduced the phosphorylation level (Fig. 5)). The I_{50} of ethylenediamine was about 0.2 mM in absence of cytoplasmic Na^+ (derived by extrapolation of a plot of the I_{50} value of ethylenediamine as a function of cytoplasmic Na^+ (inset Fig. 5) and increased strongly with the Na^+ concentrations (Fig. 5). At low Mg^{2+} and high cytoplasmic ATP concentrations ethylenediamine also strongly reduced the phosphorylation level in the absence of added Na_{cyt} and also reduced the affinity for Na_{cyt}. The $K_{0.5}$ for Na^+ under these conditions was 1 mM in absence of ethylenediamine and increased to 2 and 20 mM in presence of 2 and 20 mM ethylenediamine, respectively. Cytoplasmic Tris had no effect on the phosphorylation level in the absence of Na_{cyt} but reduced the steady-state phosphorylation level in the presence of low cytoplasmic Na^+ concentrations (Fig. 6).

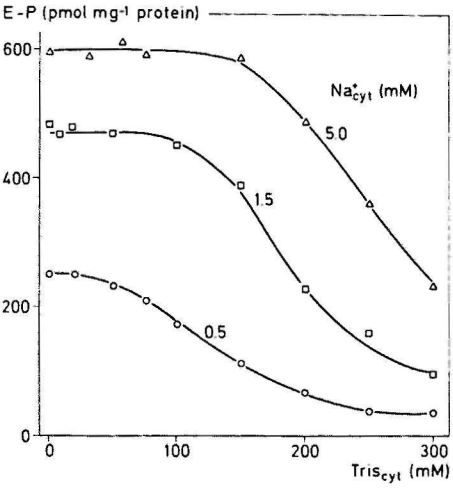

Fig. 6. The effect of cytoplasmic Tris on the steady-state phosphorylation level with different Na_{cyt} concentrations. Proteoliposomes (as in Fig. 4) loaded with 300 mM Tris (pH 7.0) were phosphorylated as described in Fig. 4. The cytoplasmic medium contained Na^+, 0.5 mM (○), 1.5 mM (□) or 5.0 mM (△) and Tris in the indicated concentrations. Sorbitol was added to the extravesicular medium to maintain the osmolarity of 540 mosM.

Discussion

Sidedness of the effect of amine compounds on the phosphorylation reaction of Na^+/K^+-ATPase

Recent work from several authors has demonstrated that amine compounds have an effect on the steady-state phosphorylation level of Na^+/K^+-ATPase [5,6,13–15]. Much has been speculated on the mechanism, but no general concept holds for the whole of these effects yet. More insight in the mechanism could be obtained by determination of the site of action of these amine compounds. Binding experiments of the amines to the enzyme could give some clues, but binding studies with ethylenediamine [12] have not elucidated this question so far.

Determination of the sidedness of action of these amines is an other possibility to get more insight in this matter. From the experiments with reconstituted Na^+/K^+-ATPase described in this paper it has become clear, that in addition to Na^+ all amine buffers tested at the extracellular side (except for histidine which has no effect at all) have a stimulatory effect on the steady-state phosphorylation level. At the cytoplasmic side, however, some amines inhibit and others stimulate steady-state phosphorylation. Amine compounds which are inhibitory in phosphorylation experiments with broken membrane sheets (ethylenediamine and Tris) appeared to be

also inhibitory at the cytoplasmic side, whereas compounds enhancing the phosphorylation reaction in open membrane preparations (imidazole and triallylamine) stimulate the phosphorylation reaction from the cytoplasmic side Histidine, which has no effect on the phosphorylation of Na^+/K^+-ATPase containing membrane sheets and on the induction of conformational changes [16 17] was also inert in the phosphorylation of reconstituted Na^+/K^+-ATPase from both sides of the membrane Thus the effect of the amines at the cytosolic side seems to be more important for the inhibitory effect in broken membrane sheets whereas the effect at the extracellular side may be the cause for the observed stimulatory effects observed in the unsided preparation

An explanation for the inertness of histidine has been proposed by Schuurmans Stekhoven et al [6] According to these authors the interaction of the positively charged amines are mediated by Coulomb forces with negatively charged groups in the enzyme The negatively charged carboxyl group of histidine, however, may cause charge repulsion with the enzyme The experiment with reconstituted Na^+/K^+-ATPase described in this paper, showing that histidine fails to stimulate at the extracellular side and is without effect at the cytoplasmic side, suggested that this explanation holds for interactions at both sides of the membrane

Mechanism of action of the amine compounds

The results of the experiments with the reconstituted Na^+/K^+-ATPase give more insight in the possible mechanisms by which the amine compounds exert their effect on the phosphorylation reaction of Na^+/K^+-ATPase Since the steady-state phosphorylation level depends upon the equilibrium between formation and hydrolysis of the phosphorylated intermediate the effect can be explained by either stimulation or inhibition of one of these two reactions

A Extracellular effects

Since the extracellular effects, when present are all stimulatory the dephosphorylation reaction must be inhibited or the phosphorylation step must be enhanced Both possibilities are considered below

(i) Inhibition of the dephosphorylation by extracellular amine compounds Two possible mechanisms for the inhibition of the dephosphorylation by amines at the extracellular side are proposed The first one is based on an observation of Nøby et al [18] that Tris inhibits the conformation change from E_1P to E_2P and by our observation [7] with reconstituted Na^+/K^+-ATPase that extracellular Tris and Na^+ seem to enhance the steady-state phosphorylation level possibly by inhibition of this conformational change From the experiments described in this paper it has become clear that most other amines share this property with Tris Alternatively the inhibition of the dephosphorylation step can be caused by screening off the K^+ sites by the extracellular amine compounds [13] In this view the amine compounds prevent the residual K^+ from binding to its dephosphorylating site and so reduce the rate of the spontaneous dephosphorylation (i e , in the absence of added K^+) This spontaneous dephosphorylation must here be seen as the reaction catalyzed by low concentrations of residual K^+ The influence of extracellular Na^+ on the dephosphorylation rate in an unsided preparation cannot be studied since it is impossible to exclude cytoplasmic effects There is no concensus in the literature on this matter Some authors reported a decrease in the dephosphorylation rate either by low concentrations of extracellular Na^+ [28] or by high Na^+ concentrations [14,29] Other authors [13,30] observed an increase in the dephosphorylation rate at high Na^+ concentrations, whereas in one study [31] no influence on the dephosphorylation rate at different Na^+ concentrations was found The influence on the dephosphorylation rate of extracellular buffers is also difficult to check, because of the low phosphorylation levels at low extracellular buffer concentrations and because of the impossibility of changing the extracellular (intravesicular) composition during the dephosphorylation reaction The dephosphorylation rate of proteoliposomes containing high and low Tris concentrations appeared to be equal This indicates that the increase of the phosphorylation level due to higher extracellular (intravesicular) Tris concentrations was not caused by accumulation of the phosphointermediate due to a slower breakdown Furthermore the turnover number of the phosphorylated enzyme was independent of the ionic composition of the extracellular medium This observation confirms the former observation that the dephosphorylation rate is not influenced by extracellular amines

(ii) Stimulation of the phosphorylation reaction Extracellular amine compounds and Na^+ can induce a conformation suited for phosphorylation (an E_1 like conformation), as already has been proposed before [5] According to results of Rephaeli et al [19], this conformation is not the conventional E_1 conformation as monitored by FITC fluorescence In their experiments with reconstituted Na^+/K^+-ATPase extracellular Na^+ had no effect on the FITC fluorescence In contrast to this observation, however, this conformation might be the same as the Na^+-form which can be monitored by eosin fluorescence [20] since ethylenediamine increases the eosin fluorescence although this compound decreases the steady-state phosphorylation level in unsided preparations [6] Further support comes from experiments with FITC-labeled enzyme An increase of the fluorescence of the labeled enzyme by ethylenediamine was observed recently [21] In addition we have observed some increase in eosin fluorescence by extracellular ethylenediamine in reconstituted Na^+/K^+-ATPase (Van der Hijden, H T W M , unpublished re-

sults) The conformation induced by extracellular amine compounds and Na_{ext} in this study is therefore supposed to be an E_1 or E_1-like conformation which needs a cytoplasmic trigger like high ATP concentrations, Na^+ or amine compounds to be phosphorylated [7]

B Cytoplasmic effects

Amines at the cytoplasmic side of the membrane show a different behaviour Some compounds like triallylamine and imidazole are stimulatory whereas others like ethylenediamine and Tris are inhibitory and competitive to Na^+ Histidine is inert with respect to the phosphorylation reaction For the stimulating compounds a mechanism as proposed by Fukushima [14] can be valid This author proposed that Na^+ and other positively charged compounds may bind to a carboxyl group of the enzyme responsible for the acceptance of a proton of a water molecule The binding of the positively charged compound prevents the formation of the hydroxyl ion as a good nucleophile for dephosphorylation This results in a retardation of the dephosphorylation rate and in an increase of the steady-state phosphorylation level An analogous concept is proposed by Schuurmans Stekhoven et al [6], who suggested that cytoplasmic amines prevent water necessary for the hydrolysis to enter the phosphate binding site These concepts, however, are not as general applicable for the reconstituted Na^+/K^+-ATPase because triallylamine and imidazole fit well in it, but Tris and ethylenediamine exert opposite effects

Another possibility is that the stimulatory amine substances trigger the phosphorylation reaction of Na^+/K^+-ATPase at the cytoplasmic side in a Na^+-like fashion [6] The inhibitory amines, however, are also supposed to bind to the Na^+ site, which can be concluded from the competitive behaviour towards Na^+, but are not able to stimulate the phosphorylation reaction as Na^+ does They inhibit phosphorylation by a direct action via this site or by preventing Na^+ binding to it

Hypothetical model of the sided action of amine compounds on the phosphorylation reaction

Rejecting the concept in which extracellular amine compounds reduce the dephosphorylation rate one can derive the following model for the action of positively charged amine compounds on the phosphorylation reaction of Na^+/K^+-ATPase Charged amines potentiate the Na^+/K^+-ATPase from the extracellular side, by induction of a E_1 or 'pre-E_1' conformational state Na^+ and amines with a suitable geometry bring the enzyme into the genuine E_1 state from the cytoplasmic side This conformation can be readily phosphorylated by ATP High concentrations of ATP and low concentrations of Mg^{2+} also stimulate the phosphorylation reaction at the cytoplasmic side Amines with a different geometry also bind to the cytoplasmic Na^+ binding site but inhibit phosphorylation by preventing Na^+ or Na^+-like cations to enter the Na^+ binding site Apart from this effect they may inhibit the dephosphorylation rate either by preventing the formation of the hydroxyl ion [14] or by the prevention of water to enter the phosphorylation site [6]

With this model results obtained from experiments with broken membrane sheets which are often confusing and difficult to interprete are more easily understood, notwithstanding the fact that the different lipid environment in both systems might have influenced the results The stimulating effect followed by an inhibiting effect of amine compounds [6] can now tentatively be explained by a combination of effects on the two membrane sides stimulation at the extracellular side and inhibition at the cytoplasmic side At low amine concentrations the stimulation is predominant, whereas at higher concentrations the inhibition dominates

Also the observation of Schuurmans Stekhoven et al [5] that imidazole can stimulate phosphorylation in the absence of Na^+, whereas Tris is not able to exert this effect, fits in this model Both amines are able to interact with the enzyme from the extracellular side Imidazole in contrast to Tris can trigger the phosphorylation reaction at the cytoplasmic side

The idea of the existence of different E_1 conformations as suggested by Schuurmans Stekhoven et al [5] is confirmed by this study In the light of the model the E_1-form which is not readily phosphorylating is the extracellularly potentiated form (pre-E_1 conformational state) whereas the phosphorylating form represents the classical E_1 conformation

That the stimulating effect of amine compounds is a general Na^+-like effect [5] can be ruled out by this study A Na^+-like effect is supposed to act at the cytoplasmic side, whereas the stimulating effects of the buffers are mainly extracellular The cytoplasmic stimulatory effects are small compared to the extracellular effects This paper shows (Figs 1 and 2) that the presence of a charged amine or Na^+ at the extracellular side is a prerequisite for a maximal phosphorylation reaction of Na^+/K^+-ATPase Therefore the observation that amine compounds increase the affinity for Na^+ in a trans fashion (at the opposite viz the extracellular membrane side [6]), can be compared with the potentiation as proposed in the model This however, cannot be the only effect of the extracellular amine because in presence of saturating Na^+ at the cytoplasmic side, only a very low steady-state phosphorylation level is obtained when insufficient amino compounds or Na^+ are present at the extracellular side The inhibitory effects of ethylenediamine and Tris are located at the cytoplasmic side, which supports the idea [6] that the inhibition by the amines is exerted via the Na^+-binding site, or a site close to it

Finally the results of this paper contribute arguments in the dispute whether free protons [14,15,22 27] or buffer substances influence the Na^+/K^+-ATPase with respect to the phosphorylation reaction in favour of the latter possibility and it is clear by now that protons as well as amine substances do exert effects on partial reactions of Na^+/K^+-ATPase.

Acknowledgements

This investigation was financially supported by the Netherlands Organization for Scientific Research (N.W O), through the Netherlands Biophysics Foundation.

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Probing the active site of transport ATPases, employing an ATP-analogue with a linear extended adenosine ring, lin-benzo-ATP.

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Submitted for publication J. Biol. Chem.

Abbreviations

Nomenclature of *lin*-benzo-nucleotides the prefix *lin* in the trivial name refers to the linear disposition of the three rings in the streched-out (by 2 4 Å) version of the adenine nucleus, "benzo" refers to the additional ring which, only when central, contains no nitrogen This terminology is adaptable to derivates similarly related to adenosine (*lin*-benzoadenosine), adenylic acid (*lin*-benzo-AMP), adenosine diphosphate (*lin*-benzo-ADP) and adenosine triphosphate (*lin*-benzo-ATP) The other parts of the names follow accepted IUPAC-IUB nomenclature The chemical name for *lin*-benzoadenosine is 8-amino-3-(ß-D-ribofuranosyl) imidazo [4,5-g] quinazoline Other abbrevations used include EGTA, ethylene glycol bis (ß-aminoethyl ether)-N,N,N',N'tetra-acetic acid, EDTA, ethylenediamine-N,N,N',N'-tetraacetic acid, MES ß-morpholino-ethanesulfonic acid, Tris, 2-amino-2-hydroxymethyl-1,3-propanediol

Probing the active site of transport-ATPases employing an ATP-analogue with a linear extended adenosine ring, *lin*-benzo-ATP.

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SUMMARY

(1) *lin*-Benzo-ATP, an ATP-analogue with a linear extended adenosine ring, is tested as substrate for renal (Na^++K^+)-ATPase and Ca^{2+}-ATPase from sarcoplasmic reticulum. (2) The affinity of (Na^++K^+)-ATPase for this substrate in Na^+-activated phosphorylation (K_m = 110 μM) and hydrolysis (K_m = 200 μM) is low compared to ATP. Both reactions can be inhibited by micromolar ouabain concentrations. (3) The affinity for Ca^{2+}-ATPase lies in the same range as for Na^++K^+-ATPase: the K_m for phosphorylation is 125 μM and for hydrolysis 385 μM. (4) For both enzymes, maximal steady-state phosphoenzyme levels are only half as high as for the native substrate. The phosphorylation reaction needs much higher Mg^{2+}-concentrations and the pH-optimum is shifted to lower values. A similar shift of the pH-optimum is observed for the hydrolysis of *lin*-benzo-ATP by both enzymes. (5) The rate of dephosphorylation of Ca^{2+}-ATPase-phosphointermediate is increased at higher pH-values. The phosphointermediate of (Na^++K^+)-ATPase is highly K^+-sensitive. (6) Addition of millimolar K^+ increases the optimal hydrolytic activity of (Na^++K^+)-ATPase with ATP, but inhibits it with *lin*-benzo-ATP as substrate. (7) The rate-limiting step of the (Na^++K^+)-ATPase cycle, the $E_2(K^+)$-E_1K^+-transition can not be induced by *lin*-benzo-ATP, which is shown by the tryptic fragmentation pattern of the α-subunit of the enzyme. (8) *lin*-Benzo-ATP does not fit in the concept of Boldyrev et al (Boldyrev, A.A. and Severin, E.S. (1984) FEBS Lett. 175, 103-106) for hydrolysis of substrates by (Na^++K^+)-ATPase. The proton-accepting properties of the corresponding nucleotide bases are not the only factor for induction of the $E_2(K^+)$-E_1K^+-transition. (9) The latter property is overruled by spatial limitations which play a role in the binding of a substrate to the low-affinity binding site. The implication of the steric restrictions are less severe for the high- than for the low-affinity substrate-binding site.

INTRODUCTION

The adenosine analogue *lin*-benzo-adenosine (8-amino-3-(ß-D-ribofuranosyl) imidazo[4,5-g]chinazoline) and the corresponding nucleotides [1,2] are characterized by the formal insertion of a butadiene unit to the centre of the purine ring system (Fig 1) as if the base would have been extended by a benzene ring. Compared with adenine this structural change leads to a linear expansion of the base part by 2 4 Å under circumstances in which all hetero atoms keep their original position. The new hetero

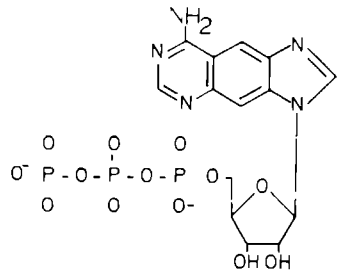

Fig 1 Structure of *lin*-benzo-ATP

atomic ring system will exhibit only slightly different properties with regard to basicity, polarizability, nucleophility and hydrophobicity This makes the synthetic *lin*-benzo-ATP a suitable substrate for the study of effects of size limitations of the substrate binding site Other than geometrical properties are hardly changed due to the extension of the adenine ring of the substrate Special attention is devoted to these analogues due to their properties as fluorophores [2] In case of the *lin*-benzo-nucleotides the anomeric conformation of the ribosylphosphate remains unchanged Since *lin*-benzo nucleotides are not commercially available, they have to be totally synthesized and cannot be prepared simply by a chemical modification of the corresponding non-extended nucleotides For principal chemical reasons this has the advantage that the synthetically prepared compounds never contain unmodified nucleotides as a possible trace contamination

The *lin*-benzo-nucleotides can be considered to act as dimensional probes to provide information concerning spatial size limitations and adaptabilities of the corresponding binding sites in enzymes Pyruvate kinase, acetate kinase and hexokinase [2] as well as mitochondrial F_1-ATPase [3] accept *lin*-benzo-adenine nucleotides nearly equally well as ADP or ATP, suggesting that the corresponding enzyme binding sites are wide enough to enable high-affinity binding of the analogues under circumstances in which also the di- or triphosphate unit can adopt the designated position to allow proper enzymatic conversion In case of F_1-ATPase, binding of *lin*-benzo-ADP leads to considerable quenching of its fluorescence intensity [3]

Since transport ATPases such as Na^++K^+- and Ca^{2+}-ATPase have adenine nucleotide binding sites exhibiting similar properties which depend on the adopted enzyme conformational states (E_1/E_2-transition), it is interesting to investigate the interaction of *lin*-benzo ATP with these enzymes It will be important to find out whether *lin*-benzo-ATP binds strongly to the remarkably specific high-affinity site of these transport ATPases and whether it is also accepted by these enzymes as a substrate analogue, i e capable of phosphorylating them In case of high-affinity binding of the analogue to these enzymes detailed fluorescence studies would provide a promising possibility for mechanistic studies Considering the current literature, high-affinity binding of *lin*-benzo-ATP does not seem to be unlikely because several chemically extended ATP derivatives such as TNP-ATP [4, 5], exhibit surprisingly high affinities to Na^++K^+-ATPase which would be consistent with a wide spatial site within the enzyme Furthermore, *lin*-benzo-ATP can also be used to probe the probably less specific low-affinity ATP binding sites [6] Binding of ATP to this site of Na^++K^+-ATPase results in a rate increase of the rate-limiting step of the overall reaction cycle [6] This step is attributed to the conformational change of the $E_2(K) \rightarrow E_1K$ transition [6] Substrate analogues other than ATP can only to a minor degree increase the rate of this transition [7] which has hitherto been explained suggestively on the basis of a reduced proton accepting ability of the N_1-adenine nitrogen in case of the analogues with respect to that of the unmodified base part [8] However, since this particular feature of *lin*-benzo-ATP is likely to be similar to ATP, the analogue is expected to act also as an interesting model compound

to test this proposed hypothesis Although a similar behaviour of Ca^{2+}-ATPase is described in the presence of substrates other than ATP [9], the proton accepting properties of the analogues appear to be less crucial

In order to get further information about the nature of the ATP binding sites of transport ATPases, the enzymatic hydrolysis of *lin* benzo ATP by renal Na^++K^+-ATPase and Ca^{2+}-ATPase from sarcoplasmic reticulum is investigated In addition to this overall reaction, relevant partial reactions such as phosphorylation, dephosphorylation and the ability of the analogue to induce the conformational E_2/E_1-transition are studied separately To achieve these aims the biosynthetic preparation of the [gamma ^{32}P]-labelled *lin*-benzo-ATP is reported here

MATERIALS AND METHODS

General chemicals and biochemicals

All chemicals used are of analytical grade, supplied by Fluka, Merck and Sigma All biosynthetically employed enzymes are from Boehringer Aqueous [$^{32}P_i$]-orthophosphate (HCl-free) is obtained from Amersham-Buchler (10 and 20 mCi) [gamma ^{32}P]-ATP is supplied by Amersham (3 Ci/mmol)

Synthesis and HPLC characterization of *lin*-benzo-nucleotides

lin-Benzo-nucleotides are synthesized according to Leonard et al [1, 2, 10, 11] For conversion of *lin*-benzo-ADP to *lin*-benzo-ATP the biosynthetic procedure employing pyruvate kinase [2] is applied with nearly quantitative yield The synthesized nucleotides are converted to the Tris salts with Dowex 50W-X4 (Tris form) and are kept in concentrated aqueous solution in liquid nitrogen Concentrations are calculated from data of photometric measurements at 260 nm based on an extinction coefficient of 18,500 cm^2/mmol at pH 7 0, as determined for *lin*-benzo-adenosine [12]

Purity of the synthesized *lin* benzo-nucleotides is analysed by HPLC (flow-rate 1 ml/min, 100 atm pressure, 25 °C) at 254 nm with a RP-18 column (ODS Hypersil, 5 µm) employing the eluent 0 3 mM tributylammonium hydrogen sulfate in 0 1 mM phosphate buffer of pH 6 0 containing 9% methanol (v/v) Retention time is 8-9 min for *lin*-benzo-ADP and 10-12 min for *lin*-benzo-ATP, the purity being higher than 98%

Synthesis of [gamma ^{32}P]-*lin*-benzo-ATP

Analogously to the synthesis of [gamma ^{32}P] ATP [13, 14] 3 5 mg D(-)-3-phosphoglycerate (tricyclohexylammonium salt), 2 4 mg NADH (Tris salt) and 20 µmol *lin*-benzo-ATP are combined with 3 25 ml of a stock solution specified below and filled up with water to a total volume of 5 ml After addition of 75 µl mercaptoethanol, 10 µl GAP-DH (10mg/ml) and 5 µl PGK (10µg/ml) each in 0 25 ml water, the reaction is initiated by adding 1 ml $^{32}P_i$ solution and allowed to proceed overnight at 25°C The stock solution (adjusted with dilute HCl to pH 8 6) contained 49 mg $MgCl_2$, 9 4 mg cysteine, 15 2 mM EGTA and 250 mg Tris in 20 ml leading to 6 mM $MgCl_2$, 2 mM cysteine, 1 mM EGTA, 50 mM Tris-HCl, 1 mM D-(-)-3-phosphoglycerate, 3 mM *lin*-benzo-ATP, 0 4 mM NADH and 100 µg GAP-DH as well as 50 µg PGK, both per ml The reaction mixture is incubated for 1 min at 80°C to denature the enzymes, immediately cooled on ice, filtered, diluted with 25 ml water and separated on a column containing 1 g Dowex 1X8 resin with 40 ml, 30 mM NH_4Cl in 0 01 M HCl, 40 ml water and 20 ml 0 25 M HCl The acidic *lin*-benzo-ATP fraction is collected under ice cooling and neutralized with cold 1 M Tris while eluting The synthesized [gamma ^{32}P]-*lin* benzo-ATP contains 42% incorporated ^{32}P and is contaminated according to the HPLC characterization with 18 ± 2% *lin*-benzo ADP (non radioactive) and 2 8 ± 0 5% $^{32}P_i$ (retention time 2 3 min in the eluent mentioned above) For carrying out most experiments described in this paper [gamma ^{32}P]-*lin*-benzo-ATP is diluted with cold *lin*-benzo-ATP resulting in a diminished *lin*-benzo-ADP and $^{32}P_i$ content (cf Discussion

under results)

Enzyme preparations

Membrane-bound Na^++K^+-ATPase is prepared from pig kidney red outer medulla according to [15], as described earlier [16] The enzymatic activity of the preparation ranges between 1800 and 2400 µmol P_i/mg h at 37°C under standard conditions [16]. Protein determination is carried out according to a modified Lowry-procedure which has been calibrated as reported elsewhere [16] The enzyme is kept on ice at a concentration of 3 mg/ml in 25 mM imidazole-HCl pH 7 5

Sarcoplasmic reticulum vesicles, prepared from rabbit skeletal muscle according to [17, 18], are made available by Prof W. Hasselbach of the Max-Planck-Institute for Medical Research, Heidelberg The Ca^{2+}-ATPase activity ranges between 140 to 200 µmol P_i/mg h at 37°C and the protein concentration is around 15 mg/ml The preparation is kept frozen in 25 mM Tris-HCl pH 7 0, 100 mM KCl and 130 mM sucrose

Phosphorylation studies

Na^++K^+-ATPase 20 µl of enzyme in buffer are mixed quickly with 80 µl of [gamma ^{32}P] *lin*-benzo-ATP in buffer of given ionic composition at 22 °C, similarly to [19] to reach the final concentration in this assay medium of 25 mM imidazole-HCl pH 7 0, 20 mM NaCl, 3 mM $MgCl_2$, 0 2 mM EDTA, 0 3 mM *lin*-benzo-ATP and 200-280 µg protein/ml In case of ATP phosphorylation studies, the substrate concentration is reduced to 1 µM and that of the enzyme to 50 µg/ml. The phosphorylation process is stopped after 10 s by addition of 3 ml 5% aqueous trichloroacetic acid (w/v) containing 100 mM phosphoric acid The denatured protein is filtered on 1 2 µm pore width Selectron filter (Schleicher & Schüll, Dassel) and washed three times with 3 4 ml stopping solution Incorporated $^{32}P_i$ is determined by scintillation counting The denatured enzyme obtained in the absence of substrate serves as a blank

Ca^{2+}-ATPase The enzyme is preincubated for 30 s at 22°C in buffer of given ionic composition before substrate is added, to start phosphorylation in a total volume of 100 µl The final concentrations in this assay medium are 25 mM MES-Tris pH 7 0, 3 mM $MgCl_2$, 0 3 mM $CaCl_2$, 0 26 mM EGTA, 0 3 mM *lin*-benzo-ATP and 0 5-1 mg protein/ml Blank values are measured in the same medium by omitting $CaCl_2$. Otherwise the procedure indicated above is applied.

Dephosphorylation studies

Na^++K^+-ATPase The phosphorylation of the enzyme by *lin*-benzo-ATP at pH 7 0 is performed as given above To 100 µl of the phosphorylation assay medium 900 µl of one of the following dephosphorylation media is added i) 25 mM imidazole-HCl pH 7 2 containing 3 mM EDTA for the investigation of the spontaneous hydrolysis of the phosphointermediate or ii) 25 mM imidazole-HCl pH 7 2 containing 3 mM EDTA and 1 mM KCl to investigate the K^+-sensitivity of phosphointermediate hydrolysis Dephosphorylation reaction is stopped after 2, 4, 6 and 8 s by acid denaturation. Filtration and counting is as described above Analogously treated samples which are acid-denatured 10 s after phosphorylation serve as a 100% control The sensitivity of the phospho-enzyme to hydroxylamine is determined as described previously [20]

Ca^{2+}-ATPase To generate sufficiently high ^{32}P-levels of the phosphoenzyme the phosphorylation is performed at pH 5 5 as specified above To 100 µl of the phosphorylation assay medium 900 µl 25 mM MES-Tris pH 5 5 or pH 7 0 containing 3 mM EDTA is added to study spontaneous phosphointermediate hydrolysis at two different pH values The sensitivity of the phosphoenzyme to hydroxylamine is also determined as described previously [20]

Enzymatic hydrolysis employing [gamma ^{32}P]-*lin*-benzo-ATP

Na^++K^+ ATPase Na^+-stimulated enzymatic hydrolysis is measured at 37°C as

release of $^{32}P_i$ from [gamma ^{32}P]-*lin*-benzo-ATP according to the method given in [28]. To 10 µl of enzyme in suspension buffer, 90 µl labelled substrate in buffer of given ionic composition is added. The standard assay medium contains 25 mM imidazole-HCl pH 7.2, 30 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 1 mM *lin*-benzo-ATP and 20-28 µg protein/ml. After an incubation time of 10 min (less than 20% hydrolysis) the reaction and thus also $^{32}P_i$-production is stopped by addition of 900 µl of an aqueous suspension of 10% charcoal in 5% trichloroacetic acid (w/v). The charcoal adsorbs *lin*-benzo-adenosine phosphates from the medium, but leaves P_i in solution. The suspension is thoroughly mixed during 10 s and left for 5 min; this procedure is repeated twice. Charcoal is sedimented by centrifugation for 10 min at 675g and 4°C. Aliquots of 0.5 ml of the supernatant are measured by liquid scintillation.

Ca^{2+}-ATPase: Ca^{2+}-activated hydrolysis of *lin*-benzo-ATP at 37°C is determined in the presence of 25 mM MES-Tris pH 7.0, 3 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.26 mM EGTA, 1 mM *lin*-benzo-ATP, 100 mM sucrose and 20 µg protein/ml. The basal, Mg^{2+}-dependent activity of the enzyme preparation is measured analogously in the absence of Ca^{2+} and is subtracted from the activity measured with Ca^{2+}. The experiments are carried out as specified above.

[gamma ^{32}P]-*lin*-benzo-ATP binding studies

Binding studies at 22°C employing a fast filtration method with correction for nonspecific nucleotide retention based on the experimentally determined [gamma ^{32}P]-*lin*-benzo-ATP content relative to that of [^{14}C]-sucrose retained in the filtrate are performed according to [21]. The binding medium contains 50 mM imidazole-HCl pH 7.0, 1 mM EDTA, *lin*-benzo-ATP concentrations in the range of 0.5 - 20 µM, 1 mM sucrose and 1 mg protein/ml. Results obtained with 10 mM non-radioactive ATP instead of the *lin*-benzo-nucleotide are used for blank subtraction.

Tryptic fragmentation in the presence of *lin*-benzo-ATP

Tryptic fragmentation [37] of Na^++K^+-ATPase is carried out by incubation of 0.67 mg/ml Na^++K^+-ATPase at 37°C in a medium containing 50 mM imidazole/HCl (pH 7.0), 0.7 mM EDTA 1 µg trypsin per ml and Na^+, K^+ and nucleotides as indicated. After stopping the digestion with trypsin inhibitor the resulting fragments of Na^++K^+-ATPase are separated by gel electrophoresis as described before [37].

Transport studies.

Na^++K^+-ATPase

Na^++K^+-ATPase was reconstituted in phosphatidylcholine/cholesterol proteoliposomes as described earlier [22]. Transport induced by ATP or *lin*-benzo-ATP was monitored by measuring the fluorescence of the membrane potential sensitive probe Oxonol VI in a thermostated cuvette holder equipped with a magnetic stirrer on a Shimadzu 510 fluorescence spectrophotometer, at excitation and emission wavelengths of 580 and 660 nm, respectively [23]. The fluorescence of 150 nM Oxonol VI in the presence of proteoliposomes was taken as F_0 and the change in fluorescence after addition of either ATP or *lin*-benzo-ATP as F_0/F_1 = delta F was recorded as the time-dependent change in the membrane potential due to active pumping of reconstituted Na^++K^+-ATPase.

Ca^{2+}-ATPase

In order to study the potency of *lin*-benzo-ATP to stimulate $^{45}Ca^{2+}$-uptake in intact endoplasmic reticulum either *lin*-benzo-ATP or ATP (3-10 mM) was added to permeabilized pancreatic acinar cells in an incubation medium set at 0.5 µM Ca^{2+}. The uptake of $^{45}Ca^{2+}$ was measured for 20 min at pH 7.4 and 37°C as described by Willems et al. [24], with additionally 20 mM oxalate present, in order to trap the accumulated $^{45}Ca^{2+}$.

RESULTS

Phosphorylation and dephosphorylation of $(Na^+ + K^+)$ ATPase by *lin* benzo-ATP

lin-Benzo-ATP is a less efficient phosphorylating substrate of $(Na^+ + K^+)$-ATPase than the native substrate ATP Within 3 s, steady-state phosphorylation levels as high as 1 2 - 1 5 nmol/mg protein can be reached, which are about half of that obtained with ATP for the same enzyme preparation (2 7 - 3 2 nmol/mg protein) The suboptimal level can partly be explained by the small contamination of the substrate with *lin*-benzo-ADP, which inhibits the phosphorylation reaction Under the phosphorylation conditions, an IC_{50} for *lin*-benzo-ADP of 0 1 mM was observed in the presence of 0 1 mM *lin*-benzo-ATP Alternatively, the reduction of the steady state phosphorylation level might be due to a lower E_1 content of the enzyme with *lin*-benzo-ATP, since this substrate cannot induce the E_2 conformational state as ATP does The affinity for the synthetic substrate is low The K_m value lies in the range of 100 - 120 μM, compared to 0 05 - 0 25 μM for ATP [19] The K_D-value for *lin*-benzo-ATP, can only be estimated on basis of a maximal binding-capacity for ATP of 3 9 nmol/mg protein in the absence of Mg^{2+} [21] Because the maximal level obtained is in the range of 95 - 125 nmol/mg protein at a *lin*-benzo-ATP concentration of 10 μM, a K_D of 0 3 - 0 4 mM is derived by extrapolation of the Scatchard plot (not shown). This value is in the same range as the above mentioned K_m value

Ouabain lowers the phosphorylation level with an IC_{50} of 5 x 10^{-6}M, the same value as determined for ATP (Fig 2) With *lin*-benzo-ATP, a level of 10% remains at the highest ouabain concentration This is due to $^{32}P_i$-phosphorylation of the ouabain enzyme-complex, which has a higher affinity for P_i-phosphorylation than the enzyme itself [25] The $^{32}P_i$-contamination originates from the synthesis of [gamma ^{32}P]-*lin*-benzo-ATP, and can not be fully removed by anion exchange chromatography

The *lin*-benzo-ATP-dependent phosphorylation needs much higher Mg^{2+}-concentrations ($K_{0.5}$ 0 2 - 0 3 mM) than the phosphorylation by ATP, where enzyme-bound Mg^{2+} (K_D=5 μM [26]) is sufficient The reason for the need for higher Mg^{2+} concentrations may be the higher substrate concentration needed for phosphorylation The half-activating Na^+-concentration lies in the range of 3-5 mM in the presence of 3 mM Mg^{2+} For ATP-phosphorylation a $K_{0.5}$ of 0 5 mM is found at 2 5 mM Mg^{2+} in imidazole-HCl, pH 7 0 and 22°C [27] A similar shift to higher Na^+ concentration was found for the half-maximal activation of the phosphorylation reaction with GTP as substrate [28]

K^+ decreases the *lin*-benzo-ATP generated phosphoenzyme level with an IC_{50} of 30 μM at 20 mM Na^+ and an IC_{50} of 50 μM at 100 mM Na^+ The *lin*-benzo-ATP-generated phosphointermediate is predominantly in the E_2P-form Its K^+-sensitivity appears to be as high as that of the ATP-generated phosphoenzyme 95% within 2 s [28] The rate of spontaneous hydrolysis, measured in the presence of EDTA is 14 4 min^{-1} for the *lin*-benzo-ATP-generated intermediate, while that of the ATP-generated intermediate lies in the range of 12-36 min^{-1} [20] Both phosphointermediates show a similar sensitivity to hydroxylamine, indicating that the same mixed anhydride was formed

The latter results indicate, that the phosphointermediate once formed by the lateral extended nucleotide *lin* benzo-ATP, is a conventional phosphoenzyme, whose properties are similar to the one formed with the natural nucleotide ATP The reaction conditions required for the maximal phosphorylation level are, however, somewhat different This is mainly due to the much lower affinity of the synthetic substrate for the enzyme Another difference is found in the pH-dependence of the phosphorylation reaction (Fig 3) The pH optimum for the phosphorylation by *lin*-benzo-ATP is shifted to lower values (pH 6 5) compared to phosphorylation by ATP At pH-values higher than pH 7 0 the *lin*-benzo-ATP generated steady-state phospho-

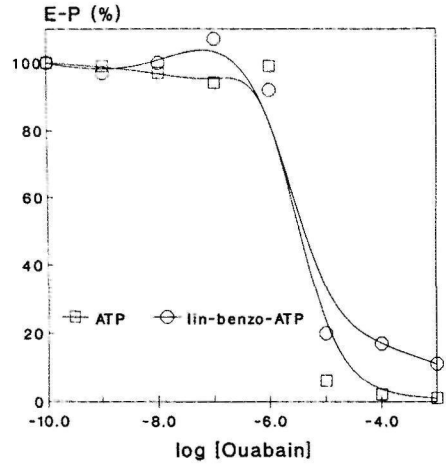

Fig. 2. Inhibition of *lin*-benzo-ATP-phosphorylation of (Na^++K^+)-ATPase by ouabain. (Na^++K^+)-ATPase is preincubated for 30 min at 22°C in a medium containing 6 mM $MgCl_2$, 25 mM imidazole-HCl (pH 7.0) and the indicated ouabain concentrations. The phosphorylation reaction with ATP (1 µM) or *lin*-benzo-ATP (30 µM) proceeds in a medium containing 25 mM imidazole-HCl (pH 7.0), 20 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EDTA for 10 sec. at 22°C. Protein concentration is 50 µg/ml in the case of ATP-phosphorylation and 200 µg/ml in the case of *lin*-benzo-ATP-phosphorylation.

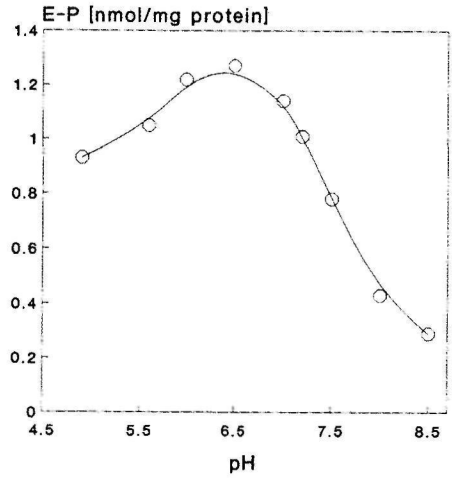

Fig. 3. Phosphorylation of (Na^++K^+)-ATPase by *lin*-benzo-ATP in dependence on pH. Phosphorylation in the presence of 125 mM MES-Tris, 20 mM NaCl, 0.2 mM EDTA, 3 mM $MgCl_2$ and 0.3 mM *lin*-benzo-ATP is allowed to proceed for 10 s at 22°C. Protein concentration is 280 µg/ml. The pH values given on the abscissa are the values determined for the applied buffer solution.

rylation levels fall to low values, while the ATP-generated levels remain nearly unchanged.

Phosphorylation and dephosphorylation of Ca^{2+}-ATPase by *lin*-benzo-ATP

The pH-dependence of *lin*-benzo-ATP-mediated phosphorylation of the Ca^{2+}-ATPase shows a similar behaviour to that found with (Na^++K^+)-ATPase: the optimum lies in the range of pH 5.5-6.2 (Fig. 4). At higher pH-values the level decreases sharply, while that of ATP phosphorylation remains constant in the range of pH 6.0-8.5 [29]. That the phosphoenzyme originates from *lin*-benzo-ATP and not from P_i, present in the *lin*-benzo ATP preparation, is shown by comparison the Ca^{2+}- and the Mg^{2+}-induced phosphorylation levels in parallel. Low pH-values drive the reaction in the direction of higher Mg^{2+}-dependent phosphoenzyme levels due to $^{32}P_i$-phosphorylation. The Mg^{2+}-dependent levels at low pH-values, however, are still low compared to the Ca^{2+}-dependent levels, generated by *lin*-benzo-ATP.

With *lin*-benzo-ATP as substrate phosphorylation levels of 1.5 nmol/mg protein can be reached at pH 5.5, which is only half of those obtained with ATP at pH 7.0 with the same enzyme preparation (2.8 - 3.1 nmol/mg protein). The level is reached within 3 s. The sensitivity to hydroxylamine indicates, that the phosphate of *lin*-benzo-ATP is bound to Ca^{2+}-ATPase to form an acyl-phosphate, like the one formed with (Na^++K^+)-ATPase. The affinity for the substrate is low: with *lin*-benzo-ATP, a K_m of 120 - 130 µM is found at pH 7.0, whereas the K_m for ATP-phosphorylation lies at 1 - 3 µM [30, 31].

The Ca^{2+}-dependence is identical for both substrates at pH 7, free Ca^{2+}-concentrations of 1 µM induce half-maximal activation [32]. The observation that high Mg^{2+} concentrations are necessary for the phosphorylation reaction with *lin*-benzo-ATP ($K_{0,5}$ = 0.2 - 0.3 mM) fits well in the concept that the true substrate is the Mg^{2+}-nucleotide-complex.

The dephosphorylation reaction of the phosphointermediate generated with *lin*-benzo-ATP is faster at higher pH-values. In a medium containing 25 mM MES-Tris and 3 mM EDTA at 22°C the rate of spontaneous dephosphorylation increases by a factor of two from 10 min^{-1} at pH 5.5 to 21 min^{-1} at pH 7.0. A similar increase of the dephosphorylation rate with increasing pH has been observed for the ATP-generated phosphoenzyme [33]. The shape of the pH-curve of *lin*-benzo-ATP phosphorylation level might be due to this pH-effect on the dephosphorylation rate.

The hydrolysis of *lin*-benzo-ATP by (Na^++K^+)-ATPase

The Na^+-dependent hydrolysis in imidazole-buffer at pH 7.2 is only a factor two different for the natural and the synthetic substrate. The maximal rate of hydrolysis is found to be 20 µmol P_i/mg protein per h with *lin*-benzo-ATP and 44 µmol P_i/mg protein per h with ATP [28]. Both activities can be totally inhibited by ouabain concentrations of 0.1 mM. The affinities of the hydrolytic reaction resemble those of the phosphorylation reaction (K_m = 200 µM for *lin*-benzo-ATP-hydrolysis at 20 mM Na^+, K_m = 0.56 µM for ATP-hydrolysis at 300 mM Na^+ [28]). The half maximal stimulatory effect of Na^+ ($K_{0,5}$ = 7 mM) is shifted to higher Na^+-concentrations for *lin*-benzo-ATP compared to that for ATP. For ATP, a $K_{0,5}$ of 0.4 mM Na^+ (in the absence of K^+) is given in the literature [38]. Mg^{2+} is essential for the reaction with a $K_{0,5}$ of 0.3 mM, compared to micromolar concentrations with ATP as the substrate. The pH-dependence of *lin*-benzo-ATP-hydrolysis shows its maximal value at lower pH-values (pH 5.5 - 6.0) than the phosphorylation reaction. A shift to lower pH-values is also observed for ATP-hydrolysis relative to the phosphorylation reaction [35].

K^+-addition at micromolar concentrations stimulates the Na^+-dependent rate of *lin*-benzo-ATP-hydrolysis (Fig. 5) to a small extent, but at concentrations higher than 0.1 mM it inhibits the hydrolytic activity of Na^++K^+-ATPase. When the effect of K^+ on Na^+-activated hydrolysis at varying

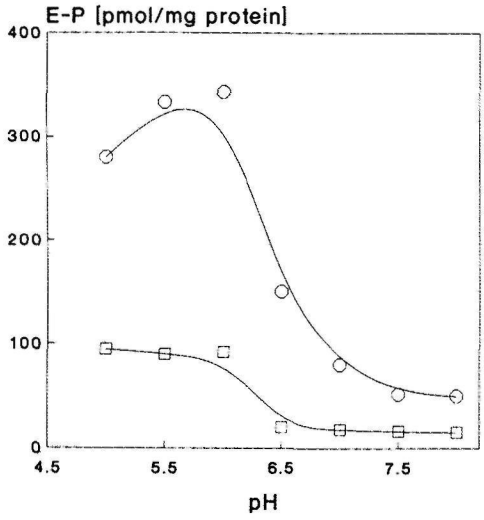

Fig. 4. Phosphorylation of Ca^{2+}-ATPase by *lin*-benzo-ATP on pH in the presence (□) or in the absence of Ca^{2+} (o). The enzyme (500 µg/ml) is incubated for 30 s at 22°C in a medium containing 125 mM Mes-Tris, 3 mM $MgCl_2$ and 0.26 mM EGTA (Ca^{2+}-free assay) or in a medium containing additionally 0.3 mM $CaCl_2$ (0.04 mM uncomplexed Ca^{2+}). The reaction is started by addition of 0.3 mM *lin*-benzo-ATP and runs for 10 sec at 22°C.

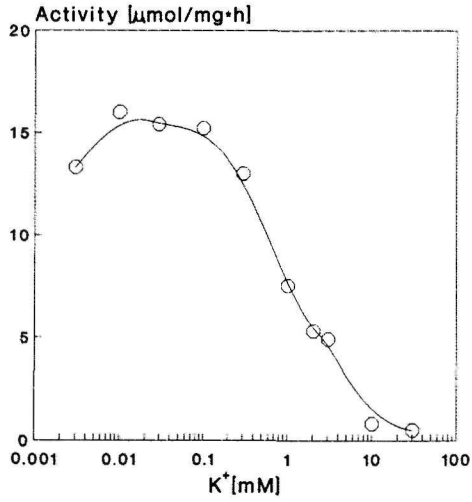

Fig. 5. Inhibition of *lin*-benzo-ATP hydrolysis of (Na^++K^+)-ATPase by increasing K^+-concentrations. The reaction runs for 10 min at 37°C in a medium containing 25 mM imidazole-HCl (pH 7.2), 30 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 1 mM *lin*-benzo-ATP and the indicated K^+-concentration at a protein concentration of 25 µg/ml.

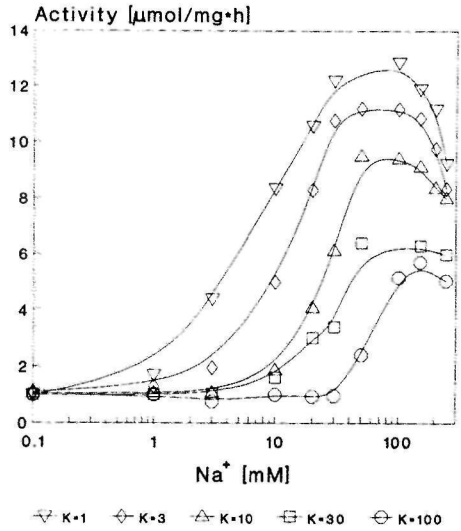

Fig. 6. Effect of Na^+ and K^+ on the hydrolysis of *lin*-benzo-ATP by (Na^++K^+)ATPase. The hydrolysis is assayed in a medium containing 25 mM imidazole-HCl (pH 7.2), 3 mM $MgCl_2$, 0.2 mM EDTA and 1 mM *lin*-benzo-ATP in the presence of indicated Na^+- and K^+-concentrations. Protein concentration is 25 µg/ml, incubation time is 10 min at 37°C.

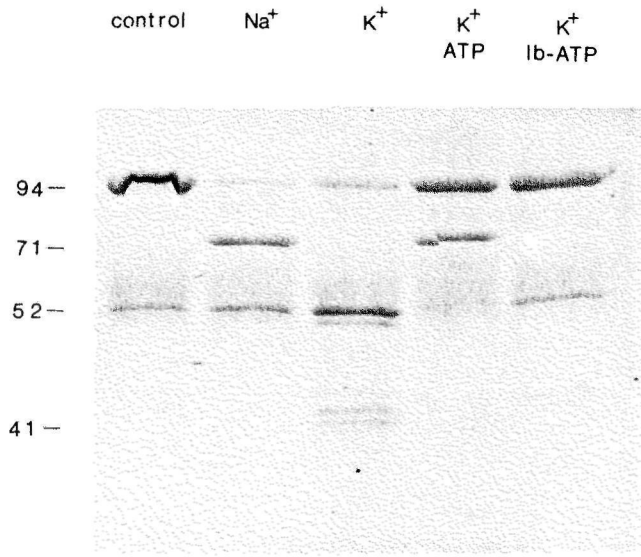

Fig. 7. Tryptic fragmentation pattern of Na^++K^+-ATPase. Tryptic fragmentation is carried out as described in Materials and Methods in the presence of 10 mM Na^+ or K^+, or 1 mM K+ plus the indicated nucleotides (5 mM). The protein bands of the polyacrylamide gel are stained with Coomassie blue. Apparent molecular weights as determined from the mobility marker proteins of known molecular weights (in kilodalton) are indicated in the left hand margin. Control is the α- and ß- subunit pattern of an unfragmented sample of Na^++K^+-ATPase.

Na^+-concentrations is studied (Fig. 6), the optimum of Na^+-activation is shifted to higher values with increasing K^+-concentrations (30 mM Na^+ in the absence and 130 mM Na^+ in the presence of 100 mM K^+). The inhibitory effect of K^+ is in contrast to the observations for ATP-hydrolysis, where a large stimulatory effect is observed after addition of millimolar K^+-concentrations.

In the presence of 20 mM K^+ the affinity for *lin*-benzo-ATP is decreased (K_m values of 200 and 625 µM were observed in the absence and presence of 20 mM K^+, respectively) and the maximal hydrolytic activity is decreased to less than half of the value measured in the absence of K^+ (from 20 µmol P_i/mg protein per h to 8 µmol P_i/mg protein per h). With ATP as substrate, two K_m-values for high- and low-affinity binding can be measured after addition of 20 mM K^+ (K_m=10 µM and 330 µM [34]). The maximal hydrolytic activity is raised by K^+ to 1800 - 2400 µmol P_i/mg protein per h.

Since the *lin*-benzo-ATP-phosphorylated intermediate is sensitive to K^+, like the ATP-phosphorylated intermediate, the inhibitory effect of K^+ above 0.1 mM must occur in a step subsequent to the dephosphorylation. A likely candidate is the E_2K-E_1K-transition, which is examined in the following section.

Nucleotide specificity of the E_2K-E_1K transition of (Na^++K^+)-ATPase as probed with tryptic fragmentation

A criterion for the conformation of the enzyme is based on the tryptic cleavage of the α-subunit of (Na^++K^+)-ATPase and the subsequent electrophoretic separation of the fragments [34, 36]. By tryptic digestion of the Na^+-stabilised E_1-conformation, a 78 kDa-fragment is formed, whereas the E_2-conformation stabilised by K^+, splits into 58 and 48 kDa-fragments (Fig. 7). Addition of ATP is sufficient for a total shift from the K^+-stabilised E_2K-conformation to the E_1-conformation, as determined by the appearence of the 78 kDa-fragment in the presence of 5 mM ATP and 1 mM K^+. Concentrations of 5 mM *lin*-benzo-ATP fail in driving this conformational change in the direction of E_1 if 1 mM K^+ is present. Tryptic digestion shows only the 58- and 48 kDa-fragments.

The hydrolysis of *lin*-benzo-ATP by Ca^{2+}-ATPase

lin-Benzo-ATP is hydrolysed by Ca^{2+}-ATPase and the reaction obeys usual Michaelis-Menten-kinetics. The K_m-value for this substrate lies in the range of 300-360 µM and reflects the low-affinity of the phosphorylation reaction. With the native substrate, two K_m-values for high- and low-affinity binding are given in the literature (K_m=1-3 µM and 50-200 µM [36]). The hydrolytic rate measured for *lin*-benzo-ATP is very low compared to the rate measured with the native substrate: with *lin*-benzo-ATP, 4-8.5 µmol P_i/mg protein per h are hydrolysed, which makes only 1-4% of the activity of the same enzyme preparation with ATP as substrate. The pH-optimum is found at pH 6.5-7.0 and is somewhat lower than that found for ATP (pH 7.4 [33]).

Transport studies

lin-Benzo-ATP (1-5 mM) had in contrast to ATP no effect on the Oxonal VI fluorescence in Na,K-ATPase containing proteoliposomes, suggesting that the pseudo substrate was not able to stimulate active transport.

lin-benzo-ATP-mediated Ca^{2+} transport into a non mitochondrial intracellular pool of permeabilized pancreatic acinar cells was only 2-5% of that found with ATP, which is in accordance with the low hydrolytic rate of the synthetic substrate.

DISCUSSION

The hydrolytic behaviour of (Na^++K^+)-ATPase with the linear extended *lin*-benzo-ATP fits well in the concept of Fu et al [28] for purine- and pyrimidine triphosphates. According to this concept, the major locus of substrate-specificity in the overall reaction mechanism in the presence

TABLE I.
KINETIC PARAMETERS DETERMINED BY BOLDYREV ET AL [8] FOR DUCK SALT GLAND (Na^++K^+)-ATPase AND DIFFERENT SUBSTRATES, COMPARED TO *lin*-BENZO-ATP HYDROLYSIS BY PIG KIDNEY (Na^++K^+)-ATPase.

Substrate	Conc. range [mM]	K_m [μM]	V [μmolP$_i$/mg.h]
ATP	< 0.4	10	800
	0.4 - 3.0	330	2000
CTP	< 0.4	20	160
	0.4 - 3.0	400	400
ITP	0.05 - 3.0	200	200
GTP	0.12 - 10	650	110
lin-benzo-ATP	0.5 - 3.3	625	8

The incubation medium used by Boldyrev et al contained 130 mM NaCl, 20 mM KCl, 4 mM imidazole-HCl pH 7.4 and 7 - 17 μg protein/ml. The hydrolysis was for 5-7 min at 37°C [8]. *lin*-Benzo-ATP-hydrolysis is measured in 130 mM $MgCl_2$ at 37°C for 10 min. The protein concentration is 23 μg/ml.

of Na^+ and K^+ is the E_2K-E_1K transition. The rate of this transition must be reduced in the presence of *lin*-benzo-ATP, as indicated by the reduction of the hydrolytic rate and the lower affinity in the presence of K^+. The result of the tryptic digestion experiment confirms the similarity with substrates which are not able to induce the E_1 conformation, like GTP [28]. The observation that for both the phosphorylation as well as for the hydrolyis reaction with *lin*-benzo-ATP more Na^+ is needed also indicates that it is difficult to get the enzyme in the E_1 conformational state when *lin*-benzo-ATP is used as the substrate.

The Boldyrev-concept [8] points out, that the proton-accepting ability of the nucleotides at the low-affinity binding site is essential for induction of the conformational change from $E_2(K^+)$ to E_1K^+. The decrease in affinity and hydrolytic rate of the Na^+- and K^+-activated cycle can be correlated with the decrease of pK_a-values of the N_1-nitrogen of purine- and the N_3-nitrogen of pyrimidine bases: adenosine 3.5; cytidine 4.2; inosine 8.8; guanosine and uridine 9.2. Although *lin*-benzoadenosine has a pK_a-value not much different form that of its natural analogue, its triphosphate is hydrolyzed very slowly by Na^++K^+-ATPase with kinetics very similar to that of ITP, GTP and UTP (Table I). ATP and CTP show complicated Michaelis-Menten-kinetics, referring to high- and low-affinity-binding of the substrates, while ITP, GTP and UTP are hydrolysed in terms of a simple hyperbolic dependence on substrate-concentration (Table I) [8].

Ca^{2+}-ATPase fits well in this comparative study: as for Na^++K^+-ATPase low-affinity binding and rate-limitation at the E_2-E_1 transition are also known for this enzyme (Table II) [30, 31]. The Boldyrev-concept, however, can not hold for Ca^{2+}-ATPase, because the hydrolysis rates of the various nucleotides can not be correlated with the pK_a-values of the corresponding bases. A low rate of hydrolysis of *lin*-benzo-ATP by Ca^{2+}-ATPase comparable to that of Na^++K^+-ATPase has been observed, indicating that the size limitation also plays an important role for this enzyme, with respect to the low-affinity substrate binding site. With Ca^{2+}-ATPase, the situation is more complicated, because a specific inhibitor for this enzyme is not available, which makes the interpretation of the results

TABLE II.
COMPARISON OF THE K_m-VALUES FOR PHOSPHORYLATION BY AND HYDROLYSIS OF *lin*-BENZO-ATP AND ATP, DETERMINED FOR (Na^++K^+)-ATPase AND Ca^{2+}-ATPase.

Enzyme	Reaction	K_m [μM] ATP		K_m [μM] *lin*-benzo-ATP
Na,K-ATPase	Phosphorylation	0.1 - 0.2	[28]	115
	Hydrolysis, Na^+-dependent	0.4	[38]	200
	Hydrolysis K^+-dependent	330 - 430	[28,35]	625
Ca^{2+}-ATPase	Phosphorylation	1 -3	[36]	100
	Hydrolysis	50 - 200	[36]	385

Conditions for reactions with *lin*-benzo-ATP are as described in previous sections and conditions for phosphorylation and hydrolysis with ATP are given in the literature cited.

more difficult. Notwithstanding, with Ca^{2+}-ATPase a quite similar situation is found, compared to Na^++K^+-ATPase and although the Boldyrev-concept is not valid for this enzyme, it is assumed that steric hindrance is the main reason for the low-affinity and the low hydrolysis rate observed with *lin*-benzo-ATP as substrate. In accordance with this we find no indication for low-affinity binding to the enzyme.

From the results it is obvious that the linear extended purine-base (prolonged by 2.4 Å through insertion of a benzene-ring in the center of the purine-ring system) does not fit in the Boldyrev series concerning (Na^++K^+)-ATPase with respect to the pK_a-value of its base, since the affinity and the hydrolytic rate are too low. In the Boldyrev concept implications of the size restrictions of the substrate are not taken into account. But since *lin*-benzo-ATP is a less efficient phosphorylating compound, not obeying the Boldyrev rules in a straightforward way, it must be concluded that apart from the proton-accepting properties of the substrate, size-limitations play an important role in low-affinity substrate binding. It must therefore, be concluded that the that the Boldyrev-concept is not sufficient to explain the substrate specificity and care must be taken when purine and pyrimidine bases are compared. Furthermore a 50% lower phosphorylation level as compared to that reached with ATP was observed with *lin*-benzo-ATP. Therefore it can not be excluded that the spatial pretentious substrate exhibits slower kinetics in the first part of the reaction cycle. Nevertheless, the kinetics of the phosphorylation and dephosphorylation reactions allow a much faster turnover of the enzyme than that observed in the presence of K^+ with *lin*-benzo-ATP as the substrate. Concerning *lin*-benzo-ATP, our hypothesis is that is binds poorly to the low-affinity site because of its extended purine ring system and thus fails in accelerating the E_2-E_1-transition. And although, the binding to the high-affinity site also occurs with lower affinity than with ATP, the implications for this substrate binding site are less severe than for the low-affinity site.

With respect to cation transport the two enzymes behave quite differently with the synthetic substrate. From the results it is clear that Na^++K^+-ATPase is not able to active electrogenic cation transport,

whereas, Ca^{2+}-ATP can transport cations with an rate similar to that of the hydrolytic reaction The question arises whether Ca^{2+}-ATPase needs the low-affinity site per se for cation transport, or that it only accelerates it For Na^++K^+-ATPase, however, low-affinity substrate binding seems to be a prerequisite for electrogenic cation transport

An overall conclusion which can be drawn is that low-affinity binding-sites of transport-ATPases have very specific demands for substrate fit. Not only the proton accepting capacity but also the steric properties play an important role in their catalytic behaviour The effect is more pronounced for the low than for the high-affinity ATP binding site

Acknowledgements

This investigation was financially supported by the Netherlands Organization for Scientific Research (NWO), through the Netherlands Biophysics Foundation. The authors would like to thank Dr Peter Willems and Mr Maarten de Jong for their help with the transport studies in permeabilized acinar cells

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The basal Mg^{2+}-ATPase activity is not part of the $(H^+ + K^+)$-ATPase reaction cycle

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Biochem. J. (in press)

Abbreviations

[1]The prefix *lin* refers to the linear disposition of the pyrimidine, benzene and imidazole rings in the "stretched out" version of the adenine nucleus in *lin*-benzoadenine, chemical name, 8-aminoimidazo[4,5-g]quinazoline, *lin*-benzo-ADP, *linear*-benzoadenosine 5'-diphosphate, *lin*-benzo-ATP, *linear*-benzoadenosine 5'-triphosphate,

Tris, tris(hydroxymethyl)aminomethane,

EDTA, ethylenediamino-N,N,N',N'-tetraacetic acid,

EGTA, bis-(aminoethyl)-glycolether-N,N,N',N'-tetraacetic acid

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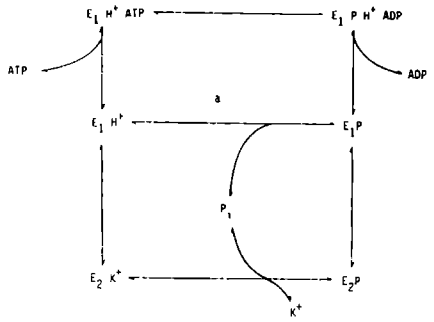
SUMMARY

Purified gastric $(H^+ + K^+)$-ATPase from the parietal cells always contains a certain amount of basal Mg^{2+}ATPase activity. *lin*-Benzo-ATP[1], an ATP analogue with a benzene ring formally inserted between the two rings composing the adenosine moiety, is an interesting substrate not only because of its fluorescent behaviour, but also because of its geometric properties. *lin*-Benzo-ATP is used in this study to elucidate the possible role of the basal Mg^{2+}-ATPase activity in the gastric $(H^+ + K^+)$-ATPase preparation. With *lin*-benzo-ATP the enzyme can be phosphorylated whereby a conventional E-P intermediate is formed. The rate of the phosphorylation reaction, however, is so slow that this reaction with subseqent dephosphorylation can not account for the much higher rate of hydrolysis of *lin*-benzo-ATP by the enzyme. This apparent kinetic discrepancy indicates that *lin*-benzo-ATP is not a substrate for the $(H^+ + K^+)$-ATPase reaction cycle. This idea was further supported by the finding that *lin*-benzo-ATP was unable to catalyze proton uptake by gastric mucosa vesicles. The breakdown of *lin*-benzo-ATP by the $(H^+ + K^+)$-ATPase preparation must be due to a hydrolytic activity which is not involved in the ion transporting reaction cycle of the $(H^+ + K^+)$-ATPase itself. Comparison of the basal Mg^{2+}ATPase activity (with ATP as substrate) with the hydrolytic activity of $(H^+ + K^+)$-ATPase using *lin*-benzo-ATP as substrate and the effect of the inhibitors omeprazole and SCH 28080 support that *lin*-benzo-ATP is not hydrolyzed by the $(H^+ + K^+)$-ATPase, but by the basal Mg^{2+}ATPase and that the activity of the latter enzyme is not involved in the H^+- and K^+- transporting reaction cycle (according to the Albers-Post formalism) of $(H^+ + K^+)$-ATPase.

INTRODUCTION

The basal Mg^{2+}-ATPase activity is a hydrolytic activity which always occurs in preparations of gastric $(H^+ + K^+)$-ATPase (1-3). This activity is, like $(H^+ + K^+)$-ATPase dependent on Mg^{2+}, but is not activated by K^+ and is assumed not to be involved in active cation transport. The basal Mg^{2+}-ATPase activity can only be partially removed from the preparation by further purification. It is, however, impossible to isolate $(H^+ + K^+)$-ATPase totally free of this basal Mg^{2+}-ATPase activity. An important question is whether this activity originates from a phosphatase which is copurified with the enzyme, or is intrinsic to the enzyme itself. In the latter case the hydrolytic activity can be due to spontaneous dephosphorylation of the phosphorylated $(H^+ + K^+)$-ATPase (Scheme 1) or to a phosphatase activity of the enzyme which is not part of the reaction cycle involved in cation transport.



Scheme 1
Modified Post-Albers Scheme of the reaction cycle of $(H^+ + K^+)$-ATPase. The a depicts a possible route for K^+-dependent P_i formation.

Experiments with substrate analogues can give important information in a study towards the mechanism of an enzyme. For ATPases, like $(H^+ + K^+)$-ATPase ATP analogues fulfil such a role (4,5). *lin*-Benzo-ATP (Fig. 1), an analogue in which the adenine ring is laterally extendend by the formal insertion of a benzene ring into the centre of the purine ring system (6,7), has been shown to be an interesting substrate for several kinases (8,9) and for mitochondrial ATPase (10), primarily because of its fluorescent properties.

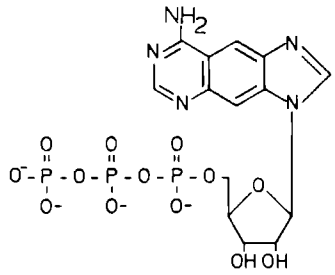


Fig.1. Structure of lin-benzo-ATP

lin-Benzo-ATP was supposed to be a proper substrate analogue for $(H^+ + K^+)$-ATPase. We therefore studied *lin*-benzo-ATP as substrate analogue for the hydrolysis and phosphorylation reaction of $(H^+ + K^+)$-ATPase. Suprisingly, the pseudo-substrate which is apart from the spatial extension equivalent to the natural substrate had a disapointingly low affinity for this enzyme in the phosphorylation reaction. On the other hand, however, it appeared to be a tool to distinguish between $(H^+ + K^+)$-ATPase and the basal Mg^{2+}-ATPase activity accompaniing the former enzyme. Surprisingly $(H^+ + K^+)$-ATPase could be phosphorylated by *lin*-benzo-ATP, but the hydrolysis of the latter substrate had to be attributed to the Mg^{2+}-ATPase activity. This apparent contradictory phenomenon has been studied in further detail in this paper.

MATERIALS AND METHODS

Preparation of the $(H^+ + K^+)$-ATPase containing vesicles and membrane sheets

$(H^+ + K^+)$-ATPase from pig stomach was prepared as described before (11) with a few modifations. Mucosal scrapings of the fundic part of the pig stomach were homogenized in a buffer containing 150 mM sucrose and 20 mM Tris/HCl (pH 7.2). The homogenates were centrifuged 20 min. at 20000xg (Sorvall SS 34 rotor). The resulting supernatant was centrifuged for 45 min. at 100000xg (MSE, 8x50 rotor). The pellet was resuspended in 25 mM Tris/HCl (pH 7.2) and centrifuged for 60 min. at 100000 g on top of a gradient consisting of 7% Ficoll/250 mM sucrose in 20mM Tris/HCl over 37% sucrose in 20 mM Tris/HCl. The buffer-Ficoll interface consisted of closed vesicles and the Ficoll-sucrose interface of broken membrane sheets. For transport studies the vesicle fraction was pelleted (60 min at 38000 rpm in a MSE 8x50 rotor). Thereafter the pellet was resuspended in buffer solution and preincubated overnight in an appropriate medium and used in the transport assay the next day. For all other assays both fractions are diluted in 20 mM Tris/HCl before centrifuging 60 min at 10000 rpm (MSE 8x50 rotor). The resulting supernatant was once again centrifuged at 38000 rpm (MSE 8x50

rotor). The four different pellets were resuspended in 20 mM Tris/HCl and frozen at -20°. After two or three freezing and thawing steps the vesicles were broken and the activities of the 10000xg-fractions ranged from 20-60 and those from the 38000 fraction from 60 to 120 µmoles per mg per h.

Synthesis of lin-benzo-ADP and lin-benzo-ATP.

The Tris salts of these components were synthesized according to the method of Leonard et al (6,9).

Synthesis of [$\gamma^{32}P$]lin-benzo-ATP

This labeled compound was prepared by the method of Glynn and Chappell (12) as modified by De Meis (13) for preparation of $\gamma^{32}P$ labeled ATP. To 3.25 ml of a reaction mixture (containing 12 mM $MgCl_2$, 4 mM cysteine, 2 mM EGTA and 100 mM Tris/HCl pH 8.6) 3.5 mg D(-)-3-phosphoglycerate (7 µmol, tricyclohexylamonium salt), 2.4 mg NADH (3.6 µmol Tris salt) and 0.2 ml 100 mM Tris-lin-benzo-ATP solution were added and a final volume of 5 ml was obtained by addition of water. The enzymatic reaction was started by addition of 75 µl 2-mercaptoethanol, 10 µl muscle glyceraldehyde 3-phosphate dehydrogenase (10 mg/ml) in 0.25 ml water, 5 µl yeast phosphoglycerate kinase (10 mg/ml) in 0.25 ml water and 1 ml $^{32}P_i$ (10 mCi in water). The reaction mixture was stirred during 16 h at room temperature. By heating the reaction mixture at 80° during 1 min the reaction was stopped which was followed by immediate cooling on ice. Subsequently the mixture was filtered (Millex-GS 0.22 µm Millipore filter) and diluted with 25 ml water. The $\gamma^{32}P$ labeled *lin*-benzo-ATP was separated by a column of Dowex-1x8 resin as described by Glynn and Chappell (12). 42% of the $^{32}P_i$ was eluted in the fraction containing the labeled *lin*-benzo-ATP. HPLC analysis (reverse phase column filled with ODS Hypersil, 5 µm) showed a contamination of 18 ± 1% *lin*-benzo-ADP and 3 ± 1% $^{32}P_i$. The contamination of *lin*-benzo-ADP is due to the settlement of the equilibrium in the enzymatic conversion. The ion exchange column was apparently not able to remove the remaining *lin*-benzo-ADP formed during the enzymatic conversion. However, in the experiments the labeled *lin*-benzo-ATP was considerably diluted with cold *lin*-benzo-ATP so that the concentration of *lin*-benzo-ADP was less than 2%.

Phosphorylation experiments

Phosphorylation of the $(H^+ + K^+)$-ATPase by *lin*-benzo-ATP and ATP was carried out at 22° at pH values as indicated in the text. The labeled *lin*-benzo-ATP and ATP concentrations were as indicated in the legends of the figures. The reaction was started by rapid mixing of 10 µl $(H^+ + K^+)$-ATPase suspension (preincubated with 0.2 mM ouabain if not indicated otherwise) with 90 µl of the medium containing ATP or *lin*-benzo-ATP and the other ligands. The reaction was stopped after 30 s (unless indicated otherwise) by addition of 3 ml 5% (w/v) trichloroacetic acid, containing 100 mM phosphoric acid. The denatured phosphoprotein was filtered on a 1.2 µm pore width Selectron filter (Schleicher and Schuell, Dassel FRG), which was then washed three times with 3 ml of the stopping solution. Incorporated ^{32}P was determined by liquid scintillation counting. For obtaining blank values experiments were carried out in which the $(H^+ + K^+)$-ATPase suspension was mixed with the stopping solution prior to addition of the substrate.

Phosphorylation of $(H^+ + K^+)$-ATPase by $^{32}P_i$ was carried out by a method similar to that of Helmich-de Jong et al (16).

Dephosphorylation

After phosphorylation for 30 s at room temperature the dephosphorylation reaction was carried out as described before (16).

The sensitivity of the phosphoenzymes to hydroxylamine was determined as described previously (36).

Hydrolysis of the substrates

The ATP hydrolysis was determined as the release of $^{32}P_i$ from [γ^{32}P] labeled ATP or *lin*-benzo-ATP (14). To 10 µl (H^++K^+)-ATPase suspension 190 µl of a medium containing cations and buffer solution, ouabain (0.2 mM) and labeled ATP or *lin*-benzo-ATP were added and this mixture was incubated at 37°. Incubation times were short (< 1 min) and the amount of P_i formed was never above 30% of that of the substrate. Several time points were taken and linearity was preserved in all cases. For obtaining blank values experiments were carried out in which (H^++K^+)-ATPase was denatured with trichloroacetic acid prior to incubation with the assay medium. The $^{32}P_i$ formation was measured after stopping the reaction at a given time by addition of 0.4 ml 10% trichloroacetic acid followed by mixing with 0.4 ml 20% (w/v) aqueous charcoal suspension. The charcoal adsorbs the adenosine phosphates from the medium, but leaves P_i in solution. The suspension was mixed thoroughly during 10 s and this was repeated after 5 and 10 min. Thereafter the charcoal was sedimented by centrifugation for 10 min at 2000 g at 4°. Aliquots (0.2-0.5 ml) were taken from the supernatant and radioactivity was measured with Cerenkov counting in a liquid scintillation analyzer.

Proton uptake measurements

The uptake of protons from the extravesicular medium was measured at 20° by recording the change in the medium pH after addition of the substrate viz. ATP or *lin*-benzo-ATP (15). Two ml of gastric vesicles (2-3 mg protein), equilibrated overnight at 4° in 5 mM glycylglycine buffer (pH 6.11) containing 150 mM K^+, were placed in a small vessel. The suspension was adjusted to pH 6.11 and was magnetically stirred throughout the experiment, while the pH was continuously recorded with a Amagruss combined electrode connected to a Radiometer PHM 75 Research pH meter with a BD 41 recorder (Kipp, Delft, The Netherlands). Measurements were started by adding known volumes of an ATP or *lin*-benzo-ATP solution adjusted to pH 6.11. Nigericin was added from a solution in ethanol to a final concentration of 10 µM.

Binding assay

Binding of [γ^{32}P] labeled *lin*-benzo ATP under non-phosphorylating conditions (free Mg^{2+}=0) has been carried out by a filtration method as described before (35). [^{14}C]sucrose was used for correction of non-specific binding to the filter. The 100 µl incubation medium contained 50 mM imidazole (pH 7.0), 3 mM EDTA, 1 mM sucrose and 2 mg protein/ml and different concentrations of labeled *lin*-benzo-ATP (0.5-30 µM). Blanks containing 10 mM unlabeled ATP were subtracted.

Materials

ATP, phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase and Tris were purchased from Boehringer, Mannheim, F.R.G. [γ^{32}P]ATP and $^{32}P_i$ were obtained from Amersham, Buckinghamshire, U.K.; nigericin and Dowex-1x8 resin from Sigma, St. Louis, MO, U.S.A.; Ficoll from Pharmacia Fine Chemicals, Uppsala, Sweden. Omeprazole was a kind gift from Dr B. Wallmark (Hässle, Mölndal), Sweden. SCH 28080 was kindly provided by Dr A. Barnett from Schering-Plough Corporation, Bloomfield NJ USA. The monoclonal antibody against (H^++K^+)-ATPase was provided by T. van Uem (Department of Biochemistry, University of Nijmegen, the Netherlands). All other chemicals were of reagent grade.

RESULTS

Formation of the phospho-intermediate by lin-benzo-ATP

Phosphorylation of $(H^+ + K^+)$-ATPase with *lin*-benzo-ATP (Fig. 1) yielded a substrate dependent phosphointermediate up to a steady state level which reached about 60% of that obtained with ATP under the same conditions (20 mM imidazole/HCl, pH 6.0, Mg^{2+} 3 mM). The steady-state phosphorylation level was not influenced by addition of 1 mM ouabain, indicating that the observed phosphorylation was not due to phosphorylation of $(Na^+ + K^+)$-ATPase present in the preparation as impurity. This suboptimal level could only partially be explained by the inhibition of the phosphorylation reaction by *lin*-benzo-ADP which was present in the substrate preparation. *lin*-Benzo-ADP inhibited the phosphorylation reaction with an I_{50} of 150 μM in presence of 300 μM *lin*-benzo-ATP. The affinity for *lin*-benzo-ATP (Fig. 2) was very low compared to that for ATP ($K_{0.5}$ 110 μM for *lin*-benzo-ATP versus 0.016-0.1 μM for ATP (16,17)). The K_d for *lin*-benzo-ATP derived by extrapolation from binding experiments with *lin*-benzo-ATP concentrations up to 100 μM was in the same range (200-400 μM). This extrapolation was based on a maximal binding capacity for ATP of 3 nmol mg^{-1} protein (18).

The most striking deviation from the characteristics of the phosphorylation reaction with ATP was the very slow rate (Fig. 3) of the phosphorylation reaction with *lin*-benzo-ATP ($k^{obs.}$ 0.1 s^{-1} for *lin*-benzo-ATP versus 23-130 s^{-1} for ATP (19, 20)). The rate limiting step in the phosphorylation reaction was not the binding of the substrate but the phosphorylation step itself. This was shown in an experiment, where the $(H^+ + K^+)$-ATPase was preincubated with *lin*-benzo-ATP in presence of EDTA and Mg^{2+} added at t=0. The rate of phosphorylation was equal to that in the experiment in which the reaction was started with *lin*-benzo-ATP (not shown). The presence of *lin*-benzo-ATP did not influence the rate of phosphorylation nor that of dephosphorylation by [γ^{32}P]ATP (not shown).

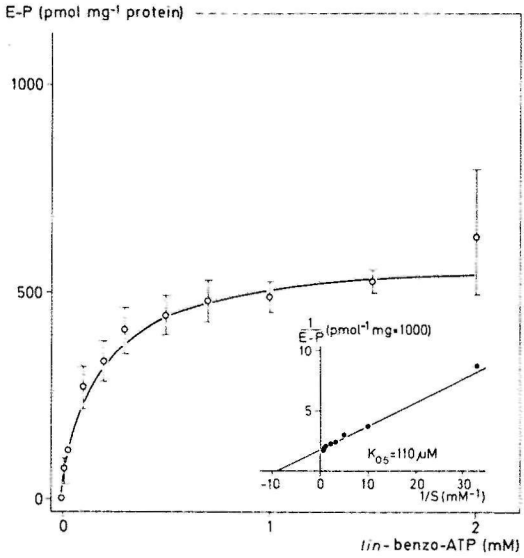
The pH optimum for the phosphorylation level by *lin*-benzo-ATP was 6.0, whereas at pH 8.0 the level was reduced to only 10%. With ATP as substrate the steady-state phosphorylation levels at pH 6.1 and 8.0 are nearly the same (31). K^+ which increased the dephosphorylation rate also reduced the steady-state phosphorylation level for *lin*-benzo-ATP with a half maximal effect at 90 μM (Fig. 4). The $K_{0.5}$ for Mg^{2+} (170 μM) in the phosphorylation reaction with *lin*-benzo-ATP was much higher than with ATP (< 5μM, ref 16), which is in accordance with the idea of a 1:1 binding of Mg^{2+} and substrate.

When phosphorylation with 1 μM [γ^{32}P]ATP was carried out after preincubation of one minute with increasing concentrations unlabeled *lin*-benzo-ATP the level of labeled phosphorylated intermediate decreased by 80% (Fig. 5A). To obtain 50% of the E-P level 50 μM *lin*-benzo-ATP was needed. This strongly suggests that ATP and *lin*-benzo-ATP phosphorylated $(H^+ + K^+)$-ATPase on the same site.

Effect of inhibitors on the phosphorylation of $(H^+ + K^+)$-ATPase by lin-benzo-ATP and ATP

Two rather specific inhibitors of the $(H^+ + K^+)$-ATPase omeprazole (21)) and the K^+-competitive SCH 28080 (22,33,34) have been used to examen their effects on the phosphorylation level of $(H^+ + K^+)$-ATPase with *lin*-benzo-ATP and ATP.

SCH 28080 decreased the steady-state phosphorylation level for both substrates with an I_{50} of 10^{-8} M. The not inhibitable level of phosphoenzyme after phosphorylation differed with the two substrates. With ATP a not inhibitable level of less than 10% remained, whereas with *lin*-benzo-ATP a rest activity of 39% was found at 10^{-6} M SCH 28080 (Table I). The cause for this different behaviour was found in the phosphorylation by inorganic phosphate. Like other P-type-ATPases $(H^+ + K^+)$-ATPase can be phosphorylated by inorganic phosphate, up to a maximal level of 2.5 nmol mg^{-1} (16,23,24). Preincubation of the enzyme with SCH 28080 increased the phosphorylation level at suboptimal P_i concentrations. A similar behaviour was found for



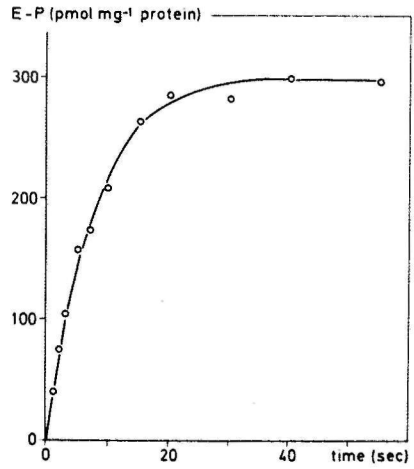


Fig. 2. (left) Substrate dependence of the steady-state phosphorylation level of (H^++K^+)-ATPase phosphorylated with lin-benzo-ATP

100 µg (H^++K^+)-ATPase was incubated at 20° in 100 µl of a medium containing 3 mM Mg^{2+}, 0.2 mM EDTA, 25 mM imidazole/acetic acid (pH 6.0) with varying concentrations of ^{32}P-labeled *lin*-benzo-ATP and stopped after 30 s with trichloroacetic acid as described in Materials and Methods. A $K_{0.5}$ value of 110 µM was obtained by Lineweaver-Burk analysis (inset). Values are given as the mean ± SD of 5 experiments in duplicate. (The solid line was drawn by eye)

Fig. 3. (right) Time dependence of the phosphorylation reaction of (H^++K^+)-ATPase with lin-benzo-ATP

(H^++K^+)-ATPase was incubated as described in Fig. 2 in presence of 100 µM *lin*-benzo-ATP. The reaction was stopped after the indicated time intervals. (The solid line was drawn by eye. A τ value of 7.45 s was calculated (program DISCRETE 1B, ref 38) for a single exponential.)

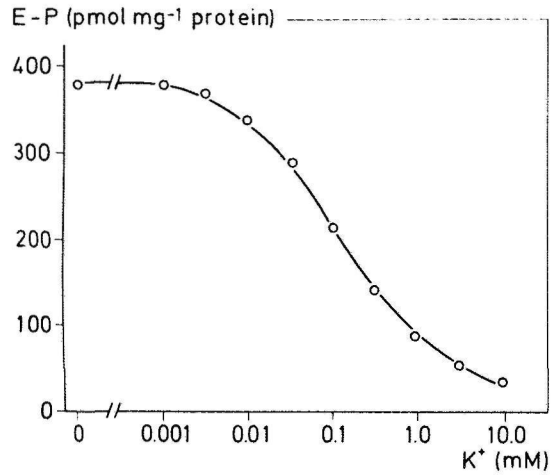


Fig. 4. K^+ dependence of the steady-state phosphorylation level

$(H^+ + K^+)$-ATPase was phosphorylated in the presence of 100 µM *lin*-benzo-ATP and K^+ concentrations as indicated. Other conditions are as described in Fig. 2. (The solid line was drawn by eye)

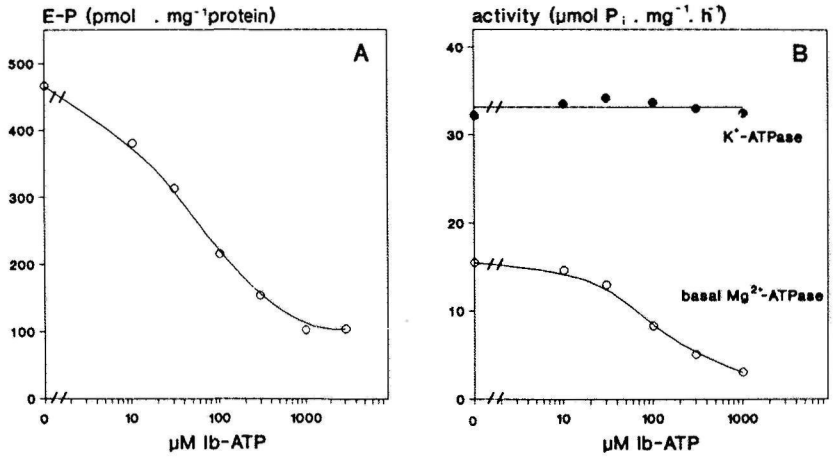
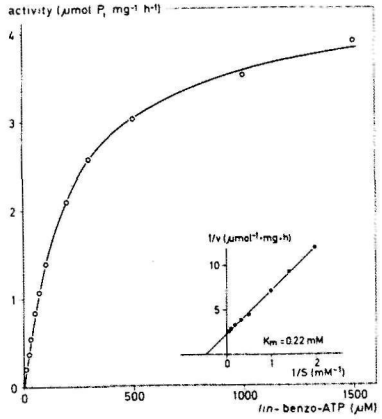


Fig. 5. Effect of lin-benzo-ATP on the steady-state phosphorylation level with ATP and the basal Mg^{2+}-ATPase and K^+-stimulated ATPase activity.

A. $(H^+ + K^+)$-ATPase (15 µg) was incubated at 22° in 100 µl of a medium containing 25 mM imidazole-acetic acid (pH 6.0), 3 mM Mg^{2+}, 0.1 mM ouabain and the indicated *lin*-benzo-ATP concentrations for one minute, whereafter, [γ^{32}P]ATP (final concentration 1 µM) was added. The steady-state phosphorylation level (E-P) was determined after 3 s incubation as indicated in Materials and Methods.

B. $(H^+ + K^+)$-ATPase (1.9 µg) was incubated for 10 min at 37°C in 200 µl of a medium containing imidazole-acetic acid (pH 7.4), 3 mM Mg^{2+}, 0.1 mM ouabain, 100 µM [γ^{32}P]ATP and the indicated *lin*-benzo-ATP concentrations in the absence (o) and presence (•) of K^+. The K^+-stimulated ATPase activity (•) was calculated by subtracting the basal Mg^{2+}-ATPase activity. The amount of ATP hydrolysis was determined as described in Materials and Methods.



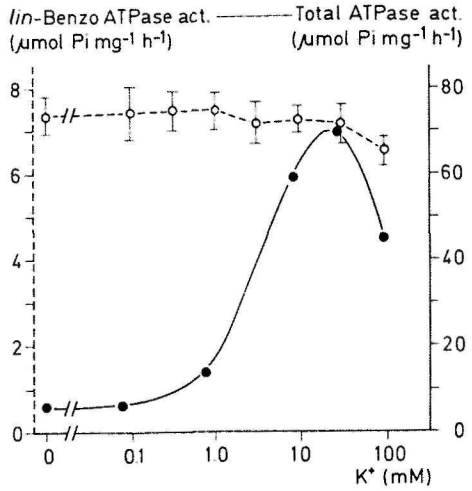


Fig. 6. (left) K_m determination of the hydrolytic activity of the (H^++K^+)-ATPase preparation.
10 µg (H^++K^+)-ATPase was incubated at 37° in 100 µl a medium containing 3mM Mg^{2+}, 0.2 mM EDTA, 25 mM imidazole (pH 7.4) and labeled *lin*-benzo-ATP in the indicated concentrations. Incubation times were chosen so that not more than 30% of the substrate was hydrolysed. The $K_{0.5}$ value of 214 µM was obtained by Lineweaver Burk analysis (inset). (The solid line was drawn by eye)

Fig. 7. (right) K^+-dependence of the hydrolysis of lin-benzo-ATP by the (H^++K^+)-ATPase preparation
10 µg (H^++K^+)-ATPase was incubated during 10 min at 37° in 100 µl of a medium containing 1 mM labeled *lin*-benzo-ATP in the presence of the indicated K^+ concentrations (o, dotted line). The symbols represent the mean ± SD of 3 experiments in duplicate. The hydrolytic activity of (H^++K^+)-ATPase with ATP under the same conditions is shown (•) for comparison. (The solid and dotted lines were drawn by eye)

	% residual steady-state phosphorylation level	
	ATP	*lin*-benzo-ATP
SCH 28080 (1 µM)	10 ± 4	39 ± 4
Omeprazole (1 mM)	<5	<5
Monoclonal AB 5B6 (100 µg/ml)	68 ± 8	70 ± 5

Table I. Residual steady-state phosphorylation level of (H^++K^+)-ATPase with ATP and lin-benzo-ATP as substrate
The (H^++K^+)-ATPase preparation was preincubated with the inhibitors SCH 28080 and Omeprazole during 30 min at 37° at pH 6.0. With the monoclonal antibodies the incubation was at 20° during 30 min. Omeprazole and SCH 28080 were disolved in a 2% DMSO solution. Omeprazole was not activated in acid medium. The phosphorylation reaction of 100 µg (H^++K^+)-ATPase was caried out at 20° in the presence of 3 mM Mg^{2+}, 0.2 mM EDTA, 25 mM imidazole/acetic acid (pH 6.0) and 0.3 mM *lin*-Benzo-ATP for 30 s. Under the same conditions 10 µg (H^++K^+)-ATPase was phosphorylated with 5 µM ATP (10 s). Values are given as mean ± SD of 4 experiments.

(Na^++K^+)-ATPase with its specific inhibitor ouabain (25). Incubation of (H^++K^+)-ATPase with 0.1 mM SCH 28080 increased the phosphorylation level with P_i by about 200%. The *lin*-benzo-ATP preparation contained about 3% inorganic phosphate, which was enough to produce the observed residual activity under these conditions. The remaining level with ATP as substrate was much smaller and could not be caused by P_i because the ATP preparation was practically free of inorganic phosphate. P_i production during the 10-s incubation period was also too small to account for this level.

Omeprazole reduced the phosphorylation level with both substrates identically. The I_{50} for both substrates was about 0.1 mM and at 1mM omeprazole only a negligable rest activity was left for both substrates (Table I). In contrast to SCH 28080 the omeprazole treated enzyme showed a decrease in the phosphorylation level with P_i in the same concentration range which inhibited the enzyme in the phosphorylation reaction with ATP. Therefore the P_i phosphorylation was neglegible with the omeprazole inhibited enzyme. The residual phosphorylation level with *lin*-benzo-ATP was practically zero.

Inhibition of the phosphorylation reaction with a monoclonal antibody 5B6 against (H^++K^+)-ATPase (Van Uem et al, unpublished) was identical for the two substrates. With ATP a rest activity of 68 ± 8% and for *lin*-benzo-ATP of 70 ± 5% was observed (Table I).

Properties of the phosphorylated intermediate

The phosphorylated intermediate formed by *lin*-benzo-ATP showed to be identical to the one generated by ATP. This phosphointermediate had a similar sensitivity to hydroxylamine as the ATP generated phosphointermediate (17), indicating that the same mixed anhydride was formed. The kinetic parameters of the dephosphorylation reaction of the phosphorylation product of *lin*-benzo-ATP matched those of the phosphorylated intermediate formed by ATP. The rate constants for both substrates were 3.0, 1.2 and >50 min^{-1} in presence of 10 mM EDTA, 10 mM EDTA + 1 mM ADP and 10 mM EDTA + 1 mM K^+ respectively. This shows that an identical K^+ sensitive phosphointermediate as with ATP has been formed.

Hydrolysis

Some of the properties of the hydrolysis reaction with *lin*-benzo-ATP matched those of the K^+-stimulated hydrolysis of ATP, but some were rather different from those of the K^+ stimulated ATPase reaction of (H^++K^+)-ATPase. The K_m for *lin*-benzo-ATP (0.22 mM; Fig 6) in the absence of K^+ for the hydrolysis reaction was comparable to that for ATP (50μM; ref 29). A small difference was observed in the need for Mg^{2+}. With *lin*-benzo-ATP (1 mM) the half maximal stimulatory effect of Mg^{2+} was at 0.2 mM, compared to 0.8 mM with ATP (3 mM) as the substrate (26).

Remarkable is the absence of the stimulatory effect of K^+ on the rate of the hydrolysis reaction. K^+ in the concentration range of 1 μM to 30 mM had no effect on the rate of hydrolysis of *lin*-benzo-ATP (Fig. 7). In the presence of 100 mM K^+ even a slight (<10%) inhibition was observed. This could be due to an ionic strength effect.

The pH dependence of the enzymatic hydrolysis reaction of *lin*-benzo-ATP did not resemble the pattern of that for ATP, but was almost identical with that of the basal Mg^{2+}-stimulated hydrolysis (Fig. 8).

The effect of *lin*-benzo-ATP on the basal Mg^{2+}ATPase activity and the (H^++K^+)-ATPase activity (with suboptimal K^+ and ATP concentrations of 1 and 0.1 mM respectively) was also studied. Fig. 5B shows that only the basal Mg^{2+}-ATPase was inhibited (I_{50}= 100 μM), whereas the K^+ stimulated activity was not affected.

When (H^++K^+)-ATPase was incubated with 3 mM Mg^{2+}, 25 mM imidazole/HCl (pH 7.4) and 1 mM *lin*-benzo-ATP in the presence or absence of K^+ the substrate was hydrolysed at a rate which varied in different preparations from 5 to 15 μmol P_i mg^{-1} h^{-1}, which is 60 to 70% of the Mg^{2+}ATPase activity, determined under the same conditions. The rate of hydrolysis of 20 different (H^++K^+)-ATPase preparations using

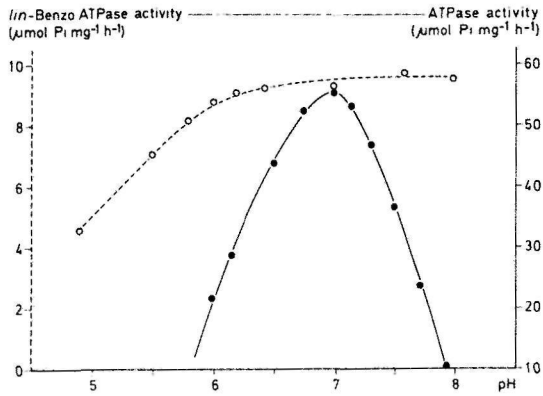


Fig. 8. Hydrolytic activity of the (H^++K^+)-ATPase preparation with lin-benzo-ATP as the substrate as a function of the pH.

(H^++K^+)-ATPase was incubated in the presence of 1 mM labeled *lin*-benzo-ATP and 50 mM MES/Tris buffer adjusted to the indicated pH under otherwise the same conditions as in Fig. 7 with 20 mM KCl(o, dotted line). The pH dependence of (H^++K^+)-ATPase with ATP (from ref 26) is added for comparison (•). (The solid and dotted lines were drawn by eye)

	% residual ATPase activity		
	K^+-stimulated ATPase	Mg^{2+}-ATPase	Hydrolysis of *lin*-benzo-ATP
SCH 28080 (10 μM)	8.5 ± 2.5	103 ± 11	100 ± 4
Omeprazole (0.1 mM)	4.5 ± 1.5	77 ± 6.0	74 ± 9
Monoclonal AB 5B6 (100 μg/ml)	28 ± 5	104 ± 9	103 ± 8

Table II. Inhibition of the hydrolytic activity of (H^++K^+)-ATPase by monoclonal antibodies 5B6 and specific inhibitors SCH 28080 and omeprazole

The hydrolytic activities were determined at 37° in the presence of 3 mM Mg^{2+}, 0.2 mM EDTA, 25 mM imidazole/HCl (pH 7.5) and 1 mM labeled substrate. For determination of the K^+-stimulated ATPase activity 3 mM K^+ was added to the medium. The hydrolytic activity with *lin*-benzo-ATP was also carried out in presence of 3 mM K^+. This did not influence the inhibition. In the experiment where the antibodies or inhibitors are used the enzyme was preincubated with the antibodies or the inhibitors for 30 min at 37°C. The hydrolysis reaction was carried out as described in Materials and Methods. The hydrolytic activity in absence of the inhibitors were taken as 100% values. Values are given as mean ± SD of 4 experiments.

lin-benzo-ATP as substrate correlated with the basal Mg^{2+}-stimulated ATP-ase activity with a correlation coefficient γ=0.94 (derived by linear regression) and much less with the K^+-stimulated (H^++K^+)-ATPase activity (γ=0.66). These findings strongly indicates that the hydrolysis of *lin*-benzo-ATP has no direct relationship with the reaction cycle of (H^++K^+)-ATPase.

Effect of inhibitors on the hydrolysis of lin-benzo-ATP

Incubation of (H^++K^+)-ATPase with 0.1 mM omeprazole resulted in an almost complete reduction of K^+ stimulated hydrolytic activity with ATP. Under identical conditions the basal Mg^{2+}-stimulated ATPase was only inhibited for 23%. The inhibition of the hydrolysis of *lin*-benzo-ATP resembled the latter one: 26% inhibition was found (Table II). After incubation with 0.1 mM SCH 28080 the K^+ stimulated hydrolysis of the (H^++K^+)-ATPase was almost completely inhibited, whereas the basal activity was not inhibited at all. Under the same conditions the hydrolysis of *lin*-benzo-ATP was neither inhibited (Table II).

In presence of the monoclonal antibody 5B6 72 ± 5 % inhibition on the K^+-stimulated ATP-ase was observed, whereas the Mg^{2+}-ATPase kept its full activity. The hydrolysis rate of *lin*-benzo-ATP remained unchanged too (Table II). The effect of the inhibitors on the hydrolysis rate of *lin*-benzo-ATP was not influenced by the presence of K^+. Thus the hydrolysis of *lin*-benzo-ATP by the (H^++K^+)-ATPase preparations matched the properties of the Mg^{2+}-ATPase activity rather than those of the K^+-stimulated activity.

Proton transport

The active transport of protons into gastric vesicles preloaded with K^+ is due to the exchange of K^+ against protons by the pumping activity of the (H^++K^+)-ATPase. After addition of ATP to a vesicle suspension the active uptake of protons could be monitored by an increase of the medium pH by use of a sensitive pH electrode. Upon addition of the ionophore nigericin the medium pH dropped fastly a few minutes after the addition of ATP as a result of protons leaking out of the vesicles. In presence of vanadate no pH jump apart from a small pH artefact was observed upon addition of ATP.

Upon addition of several concentrations of *lin*-benzo-ATP (0.1-1.0 mM) to the medium containing gastric vesicles no active pumping could be registred. Only a small artefact similar to that for ATP in presence of vanadate was observed. In presence of vanadate or nigericin the artefact remained exactly the same. Addition of nigericin after adddition of *lin*-benzo-ATP had no effect on the measured pH, indicating that no protons were accumulated in the vesicle.

DISCUSSION

It is an old dispute in the field of gastric ATPase whether the basal Mg^{2+}-stimulated ATPase activity is an intrinsic part of the (H^++K^+)-ATPase reaction cycle (Scheme 1), or is due to an impurity in the enzyme preparation. An argument for the second possibility is the reduction of the Mg^{2+}-ATPase activity upon further purification by zone- or free flow electrophoresis (26,27). The observation that the Mg^{2+}-ATPase activity and the (H^++K^+)-ATPase activity were imunologically different (28) also supports the latter possibility. The observation of Wallmark et al (29), however, that the rate of spontaneous dephosphorylation is fast enough to cause the basal hydrolytic activity of the enzyme and the fact that it is impossible to discard the basal activity totally from the enzyme preparation rather support the first possibility. The fact that the amount of basal Mg^{2+}-ATPase increases upon aging of the enzyme could also be an argument in favour of the first possibility. In this view the basal activity could originate from partially inactivated enzyme. The effects of treatment of a (H^++K^+)-ATPase preparation with phospholipase A_2 viz. a stimulation of the basal Mg^{2+}-ATPase activity together with a reduction of the K^+-stimulated ATPase activity (30) argue also in favour of the first possibility.

With *lin*-benzo-ATP we have a tool which can distinguish between the two activities, K^+-stimulated ATPase and basal Mg^{2+}-stimulated ATPase. This substrate is hydrolysed well by the Mg^{2+}-stimulated ATPase, whereas it is no substrate for the K^+ stimulated ATPase. On the other hand, *lin*-benzo-ATP can phosphorylate the $(H^+ + K^+)$-ATPase, although the rate of phosphorylation is very slow.

The product of phosphorylation of $(H^+ + K^+)$-ATPase by *lin*-benzo-ATP strongly resembled the phospho-intermediate generated by ATP. The differences like the lower affinity for the substrate and the higher $K_{0.5}$ value for Mg^{2+} are likely to be due to the different pK_a value and steric hindrance of the extended adenosine ring of the synthetic substrate. It is remarkable that a slight modification of the adenosine ring system (an extension of 2.4 Å) has such severe implications for the affinity to the enzyme. The lower steady-state phosphorylation level obtained with *lin*-benzo-ATP compared to that of the natural substrate was only partly due to substrate inhibition, but is more likely due to the slow phosphorylation rate, which is in the same range as that of the dephosporylation step so that a maximal level cannot be reached.

The extreme slow rate of phosphorylation of *lin*-benzo-ATP (200 times lower than with ATP (19, 20)) made it kinetically impossible, that the observed rate of hydrolysis was due to the phosphorylation and subsequent dephosphorylation of $(H^+ + K^+)$-ATPase. The observed initial velocities of the phosphorylation reactions indicate that maximally 4% of the P_i formed could be attributed to phosphorylation and subsequent dephosphorylation of $(H^+ + K^+)$-ATPase. The argument of Wallmark et al (29) that the dephosphorylation reaction in absence of K^+ is fast enough to be responsible for the basal hydrolysis (and therefore the basal activity may be an intrinsic one) of ATP only holds for low ATP concentrations and for preparations with low amounts of basal Mg^{2+}-ATPase activity.

The formation of a steady state phosphorylation level, however, demands that the rate of dephosphorylation is slower than that of the phosphorylation reaction. A strong increase in the rate of dephosphorylation by addition of K^+ indeed reduced the steady-state phosphorylation level but had no effect at all on the rate of hydrolysis of the synthetic substrate. This means, that in the presence of K^+, when the dephosphorylation reaction is not rate limiting, the rate of the phosphorylation must be the slowest step in the cycle. Because it is very unlikely that the phosphorylation rate will increase in the presence of K^+ (31), only a minor part of the hydrolysis of *lin*-benzo-ATP could be due to the action of $(H^+ + K^+)$-ATPase via the E-P cycle. The major part >95% of the hydrolysis of *lin*-benzo-ATP must be due to other hydrolysing activity.

Further evidence for the latter hypothesis comes from experiments with inhibitors, where the inhibition pattern of the hydrolysis matched that of the Mg^{2+}-stimulated ATPase and not that of the K^+-stimulated ATPase. In addition the pH dependence of the hydrolysis of *lin*-benzo-ATP was very similar to that of the Mg^{2+}-ATPase activity (26) and did not resemble that of the K^+-stimulated ATPase activity. The fact that *lin*-benzo-ATP inhibits the basal Mg^{2+}-ATPase but not the K^+ stimulated ATPase activity, the positive correlation between the hydrolysis of *lin*-benzo-ATP and the Mg^{2+}-ATPase activity and the lack of transport capacity are further arguments, that the hydrolysis of *lin*-benzo-ATP is catalysed by the Mg^{2+}-ATPase. Finally the Mg^{2+} dependence with *lin*-benzo-ATP as substrate is a strong indication that the phosphorylated intermediate is not responsible for the observed rate of hydrolysis. Phosphorylation with *lin*-benzo-ATP needs more Mg^{2+} than the hydrolysis, whereas the opposite is true for phosphorylation and hydrolysis with ATP as substrate.

Wallmark et al (29) found a biphasic substrate dependency for the basal Mg^{2+} ATPase activity with $K_{0.5}$ values of 0.4 and 50 µM. They cannot distinguish between two ATP sites on one enzyme or on two different enzymes. We found with *lin*-benzo-ATP only one K_m value for the substrate. Supposed that the high affinity site for ATP is due to $(H^+ + K^+)$-ATPase and the low affinity one to the basal Mg^{2+}-ATPase activity, then the affinity of the latter site for *lin*-benzo-

ATP is only 5 times lower than for ATP, whereas the difference in affinities for the phosphorylation site is more than 200 This is in line with the idea that the basal Mg^{2+}-ATPase activity has a less specific substrate site

The dispute whether the Mg^{2+}-ATPase activity is due to another protein copurified with $(H^+ + K^+)$-ATPase or to catalysis of the hydrolysis of the substrate by a reaction apart from the reaction cycle of the $(H^+ + K^+)$-ATPase itself cannot be answered by this study The characteristics of the basal Mg^{2+}-ATPase activity described in this report is definitely not the same as the Mg^{2+}ATPase in the low density membranes as described by Nandi et al (37) The separation of an impurity which was Ca^{2+} and Mg^{2+} dependent ATPase from a $(H^+ + K^+)$-ATPase preparation has been reported (32) Testing the properties of this impurity with *lin*-benzo-ATP would further elucidate this question

Acknowledgements

The authors would like to thank Mr G Schimmack for the synthesis of *lin*-benzoadenosine, Dr H Ruf for his help in some of the quantitative evaluations Mr H Swarts for technical assistance and Mr T. van Uem for the monoclonal antibody against $(H^+ + K^+)$-ATPase This investigation was financially supported by the Netherlands Organization for Scientific Research (N W O) through the Netherlands Biophysics Foundation

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Demonstration of the electrogenicity of proton translocation during the phosphorylation step in gastric (H^++K^+)-ATPase

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J. Membr. Biol. (in press)

Demonstration of the electrogenicity of proton translocation during the phosphorylation step in gastric H,K-ATPase

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Abstract

Membrane fragments containing the H,K-ATPase from parietal cells have been adsorbed to a planar lipid membrane. The transport activity of the enzyme was determined by measuring electrical currents via the capacitive coupling between the membrane sheets and the planar lipid film. To initiate the pump currents by the ATPase a light driven concentration jump of ATP from caged-ATP was applied as demonstrated previously for Na,K-ATPase (Fendler, K., Grell, E., Haubs, M., Bamberg, E., 1985. EMBO J. 4: 3079-3085). Since H,K-ATPase is an electroneutrally working enzyme no stationary pump currents were observed in the presence of K^+. By separation of the H^+ and K^+ transport steps of the reaction cycle, however, the electrogenic step of the phosphorylation could be measured. This was achieved in the absence of K^+ or at low concentrations of K^+. The observed transient current is ATP dependent which can be assigned to the proton movement during the phosphorylation. From this it was concluded that the K^+ transport during dephosphorylation is electrogenic, too, in contrast to the Na,K-ATPase where the K^+ step is electroneutral. The transient current was measured at different ionic conditions and could be blocked by vanadate and by the H,K-ATPase specific inhibitor omeprazole. An alternative mechanism for activation of this inhibitor is discussed.

Key words: gastric H,K-ATPase, caged ATP, pump currents, planar lipid films.

Introduction

The H^+ and K^+ transporting ATPase from parietal cells in the gastric mucosa (H,K-ATPase EC 3.6.1.36) is an electroneutral ion pump, which exchanges protons for potassium ions (Forte et al., 1967, Lee et al., 1974, Sachs et al., 1976). The physiological role of the pump is to produce and to maintain a pH level of about 1 in the lumen of the mammalian stomach. The H,K-ATPase is located in the secretory membrane system of the parietal cell, where the protons must be transported against a gradient of 6 pH units. Transport activity of the H,K-ATPase in membrane liposomes or reconstituted proteoliposomes has been described previously. The energy for the active hydrogen transport against the enormous pH difference is obtained by ATP hydrolysis, with a stoichiometry of 1 to 4 H^+/1 ATP depending on the source (Lee et al., 1974, Sachs et al., 1976, Reenstra & Forte, 1981, Rabon et al., 1982, Smith & Scholes, 1982, Skrabanja et al., 1984, 1987). From conformational studies it has been deduced that the H,K-ATPase behaves like an E_1,E_2 type enzyme (Schrijen et al., 1980, 1981, Wallmark et al., 1980, Morii et al., 1984, Jackson et al., 1983, Helmich-de Jong et al., 1987) as has been proposed for the ubiquitous Na,K-ATPase by Albers and Post (Albers, 1967, Post et al., 1969). Na,K-ATPase and the H,K-ATPase have a homology of more than 60 percent in the primary structure (Shull et al., 1985, Shull &

Lingrel, 1986, Shull & Greeb, 1988) The Na,K-ATPase, however, is an electrogenic pump with a stoichiometry of $3Na^+$ to $2K^+$ The electrogenicity of the Na,K-ATPase was directly demonstrated in intact cells (Thomas, R C, 1969, Abercrombie & De Weer, 1978, Gadsby et al, 1985, Lafaire & Schwarz, 1984), in reconstituted proteoliposomes (Goldin & Tong, 1974, Goldschlegger et al, 1987), or in planar lipid membranes (Fendler et al, 1985) by measuring the stationary electrical pump current or by use of voltage sensitive dyes in case of the reconstituted proteoliposomes The H,K-ATPase, however, as an electroneutral exchanging enzyme is not expected to elicit any stationary current, because the Albers-Post scheme predicts for the H,K-ATPase a phosphorylation step concomitant or prior to the proton translocation, followed by dephosphorylation concomitant with the potassium ion translocation The sum of these steps represents the electroneutral exchange across the membrane, meaning that both the proton translocation and the K^+ step are electroneutral, or that both steps are electrogenic, but oppositely directed Recently, voltage sensitivity of the K limb of the reaction cycle was shown on H,K-ATPase containing vesicles (Lorentzon et al, 1988)

Planar lipid membranes are an excellent tool by which to study the electrical properties of ion pumps This was demonstrated for the first time for the light driven proton pump bacteriorhodopsin by Skulachev and his associates (Drachev et al, 1974, 1976 a,b) The method consists of the adsorption of bacteriorhodopsin proteoliposomes or of bacteriorhodopsin-containing purple membranes to a planar lipid bilayer, where either photopotentials or photocurrents are obtained via the planar lipid bilayer as a capacitive electrode A quantitative description of the system for proteoliposomes and purple membranes adsorbed to the lipid film has been given (Herrmann & Rayfield, 1978, Bamberg et al, 1979)

The immediate application of light as the energy supplying substrate synchronizes the activation of bacteriorhodopsin, permitting detailed studies of the stationary and kinetic properties of the pump currents to be performed (Dancshazy & Karvaly, 1976, Herrmann & Rayfield, 1978, Bamberg et al, 1979, Fahr et al, 1981) Since for ATP driven pumps like the Na,K-ATPase or the Ca^{2+} ATPase from sarcoplasmic reticulum the time required to add ATP by mixing is at least a few seconds, the method described above cannot be applied A photolabile analogue of ATP, caged ATP, which produces a concentration jump of ATP in the millisecond range following irradiation with u v -light (Kaplan et al, 1978, McCray et al, 1980) was successfully applied to the Na,K-ATPase, Ca^{2+}ATPase from sarcoplasmic reticulum and to the F_o, F_1 ATP synthase from photosynthetic bacteria on planar lipid films By adsorption of membrane fragments (Na,K-ATPase), membrane vesicles (Ca^{2+}ATPase from sarcoplasmic reticulum) or reconstituted proteoliposomes (F_o, F_1 ATP synthase) to a planar lipid film, the properties of the electrical pump currents were investigated (Fendler et al, 1985, Nagel et al, 1987, Fendler et al, 1987, Borlinghaus et al, 1987, Hartung et al, 1987, Christensen et al, 1988)

Based on these studies we applied this technique to gastric H,K-ATPase Here we show for the first time the generation of transient electrical currents by a pump that operates electroneutrally under stationary conditions This was achieved by separation of the pump cycle into its phosphorylation phase and its dephosphorylation phase, respectively

A preliminary report on these results was presented at the 5th International Conference on Na,K-ATPase, Fugslo, Denmark 1987, and has been published (Fendler et al, 1988)

Materials and Methods

Preparation of the H,K-ATPase membrane sheets

H,K-ATPase from pig stomach was prepared as previously described (Forte et al, 1974, Rabon et al, 1985) with a few modifications Mucosal scrapings of the fundic part of the pig stomach were homogenized in a buffer containing 150 mM sucrose and 20 mM Tris/HCl (pH 7 2) The homogenates were centrifuged for 20 min, at 20000 x g (Sorvall SS 34 rotor) yielding a supernatant that was centrifuged for 45 min at 100000 x g (MSE, 8x50 rotor) The resulting pellet was resuspended in 25 mM Tris/HCl (pH 7 2) and centrifuged for 60 min at 100000 x g on top of a gradient consisting of 7% Ficoll/250

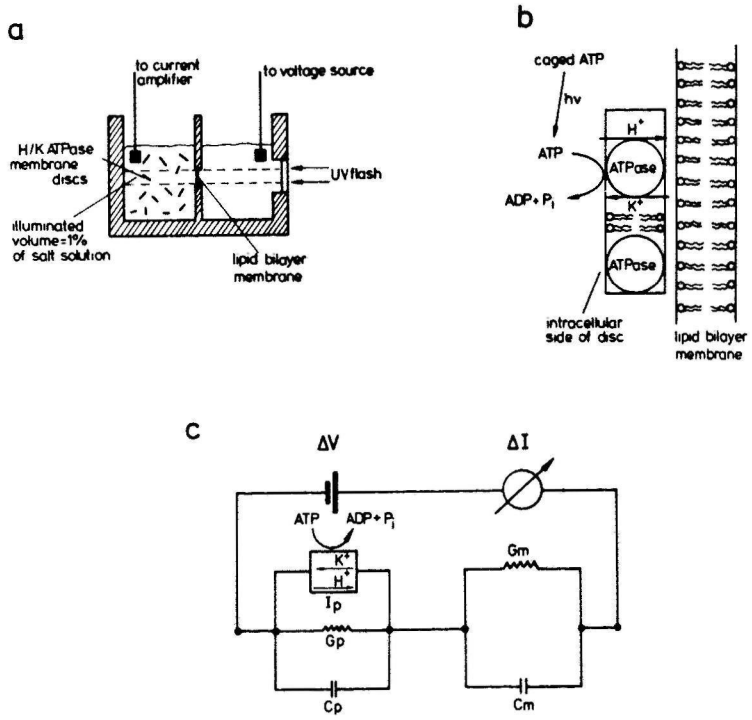

Fig. 1
Schematic representation of the bilayer setup
a) Teflon cell with black lipid membrane and adsorbed H,K-ATPase membrane fragments.
b) proposed sandwich-like arrangement of discs and underlying lipid membrane. It has to be noted that in experiments with ionophores incorporation of ionophores into the membrane fragments cannot be excluded (not shown in the figure).
c) equivalent circuit diagram of the two membranes in series. G_m and G_p refer to the conductance of the planar film and the membrane fragments, respectively. C_m and C_p to the corresponding capacitances. I_p designates the pump current generator.

mM sucrose in 20 mM Tris/HCl (pH 7.2) over 37% sucrose in 20 mM Tris/HCl (pH 7.2). The buffer-Ficoll interface consisted of closed vesicles and the Ficoll-sucrose interface of broken membrane sheets. Both fractions were diluted in 20 mM Tris/HCl (pH 7.2) before centrifuging 60 min. at 10000 x g (MSE 8x50 rotor). The resulting supernatant was once again centrifuged in 20 mM Tris/HCl (pH 7.2) at 38000 x g (MSE 8x50 rotor). The four different pellets were resuspended in 20 mM Tris/HCl (pH 7.2) and frozen at -20°C. After two or three freezing and thawing steps the vesicles were broken, yielding activities ranging from 0.02-0.06 for the 10000 x g-fractions and from 0.06 to 0.12 mmoles per mg per hour for the 38000 x g fractions. In some experiments further purification was carried out by zonal electrophoresis (Walters & Bont, 1979). Both preparations of membrane sheets gave similar results on planar lipid membranes. Residual activity of Na,K-ATPase in the preparation was suppressed by the addition of 1 mM ouabain to the membrane sheets prior to their addition to the planar lipid membranes. Enzymatic activity was determined in the presence of 0.0375 mg enzyme per ml and 5 mM Tris-ATP at 37°C employing the technique of Jørgensen, 1974, with incubation times of 8 and 15 minutes. The enzymatic activity of the gastric H,K-ATPase is calculated as the difference between the activities determined in a medium containing 60 mM imidazole-HCl, 10 mM $MgCl_2$, 40 mM KCl and 0.2 mM ouabain pH 7.5 and in a similar medium where KCl is replaced by 40 mM choline chloride. The activity of the investigated sample was 27 µmol P_i mg^{-1} h^{-1}. The Tris salts of caged ATP and ATP were prepared employing a Dowex 50 W ion exchanger column.

Membrane setup

Optically black lipid membranes with an area of $10^{-2}cm^2$ were formed in a thermostated Teflon cell with 1.5 ml of an appropriate electrolyte solution in each compartment (Mueller et al., 1964). The membrane-forming solution contained 1.5% phosphatidylserine w/v in n-decane or 1.5% w/v diphytanoyllecithin together with 0.025% w/v octadecylamine. To improve the adsorption of the negatively charged membrane sheets to the supporting lipid bilayer made from phosphatidylserine, about 10 µM free Ca^{2+} was added to the electrolyte. For the same purpose octadecylamine was added to the lipid forming solution in case of diphytanoyllecithin yielding a positive surface charge on the lipid bilayer (Dancshazy & Karvaly, 1976).

The membrane was connected to an external measuring circuit via Ag/AgCl electrodes. To avoid artificial photoeffects the electrodes were separated from the aqueous compartments of the cell by agar-agar salt bridges. To prevent light pipe effects, the salt bridges were made from polyethylene tubing. In addition, the agar-agar in the bridges contained black ink to avoid any light conduction to the electrodes.

Caged ATP was photolysed by an u.v.-light pulse of 125 ms duration time with a maximal light intensity of 3.7 W/cm^2. The light intensity could be attenuated with appropriate u.v.- light filters (Melles-Griot, Darmstadt, FRG). As light source a mercury high pressure lamp was used (200 W). Unless otherwise indicated, light pulses were applied in intervals of 10 min. After each measurement the stirrers in the cuvette were turned on, so that the liberated ATP was diluted within the path of the light beam and hydrolyzed by the enzyme in suspension. Consequently, the original concentration of caged ATP at the membrane surface was nearly recovered. Each flash liberated about 0.3 % of the total amount of caged ATP in the cuvette (Nagel et al., 1987). Knowing the geometry of the light beam in the cuvette, the conversion rate of caged ATP was determined using a luciferin-luciferase assay (Fendler et al., 1985, Nagel et al., 1987). A schematic representation of the experimental situation is given in Fig.1. The two membranes in series are capacitatively and DC coupled, in the absence and the presence of the ionophores, respectively. The pump current can be described as follows (Bamberg et al., 1979):

$$I(t) = I_\infty + (I_0 - I_\infty)\exp(-t/\tau) \qquad (1)$$

$$I_0 = I_{p0}\,\frac{C_m}{C_m + C_p} \qquad (2)$$

$$I_\alpha = I_{p0} \frac{G_m}{G_m + G_p + \frac{I_{p0}}{V^*}} \quad (3)$$

$$\tau = \frac{C_m + C_p}{G_m + G_p + \frac{I_{p0}}{V^*}} \quad (4)$$

where I_α is the current at $t \rightarrow \infty$, I_0 is the initial current at $t = 0$, I_{p0} is the pump current and V^* a constant. G_m, G_p are the conductances of the planar film and the adsorbed membrane sheets, respectively, C_m, C_p are the corresponding electrical capacitances. τ is the RC time of the system, which depends on the pump current I_{p0}. The discharging time is given by

$$\tau_{off} = \frac{C_m + C_p}{G_m + G_p} \quad (5)$$

which is equal to $\tau(I_{p0} \rightarrow 0)$.

Chemicals

P^3-1-(2-nitro)phenylethyladenosine-5-triphosphate (caged ATP) was prepared according to Kaplan et al., 1978 and Fendler et al., 1985. Phosphatidylserine from bovine brain and synthetic diphytanoyllecithin were purchased from Avanti Lipid Products, Birmingham, Al. USA. The luciferin luciferase assay kit was obtained from Boehringer, Mannheim, FRG. DTT (dithiothreitol) was obtained from Roth, Karlsruhe, FRG. The u.v.-light-insensitive protonophore 1799 (1,1,1,7,7,7-hexafluoro-2,6 - bis (trifluoromethyl) heptane-4-one was kindly provided by Dr. P. Heytler, DuPont de Nemours, Monensin was a gift from Dr. G. Szabo, Galveston, TX, USA. Omeprazole was a gift from B.Wallmark, Mölndal, Sweden. All other reagents were analytical grade or suprapur standard (Merck, Darmstadt, FRG).

Results

Demonstration of ATP dependent currents

H,K-ATPase membrane discs were added together with caged ATP under stirring to one side of the lipid bilayer membrane. After 20 min the first u.v.- light flash was applied in order to produce a concentration jump of ATP from caged ATP. Under physiological ion conditions (20 mM K^+, 120 mM Na^+, 3 mM Mg^{2+}, 50 mM imidazole (pH 7.0)) no electrical response was observed at all. A transient biphasic electrical current, however, was detected when K^+ was absent, indicating a charge movement during the phosphorylation process (Fig. 2 trace a). The sign of the transient current was the same in all experiments. The first phase corresponded to a movement of positive charges towards the protein-free side of the planar lipid membrane. The oppositely-directed current phase disappeared after the addition of the proton conducting system 1799 plus monensin, so that this component may represent the discharging of the membrane capacitors governed by the RC time constant (see Materials and Methods). The u.v.- insensitive protonophore 1799 together with the H^+, Na^+ exchanging carrier monensin was applied in previous work for the Na,K-ATPase in order to increase the electrical permeability of the underlying lipid film in the sandwich-like structure as shown in Fig. 1 (Fendler et al., 1985, Nagel et al., 1987). This was necessary for the demonstration of the stationary pump currents. Trace b in Fig. 2 shows that in the presence of the ionophores H,K-ATPase does not produce a stationary pump current. Successive additions of K^+, which increases the speed of dephosphorylation, decreased the peak current but did not create stationary currents (data not shown), reflecting the electroneutral exchange of potassium ions and protons by the pump. The peak current disappears after addition of 50 mM K^+ which can be explained by the kinetics of the pump cycle (see below). The ATP- induced current is abolished completely by the addition of vanadate (trace c in Fig. 2). The blockade by vanadate occurs only when the agent is added to the protein-containing side of the membrane. Successive additions of caged ATP and vanadate to the protein free side had

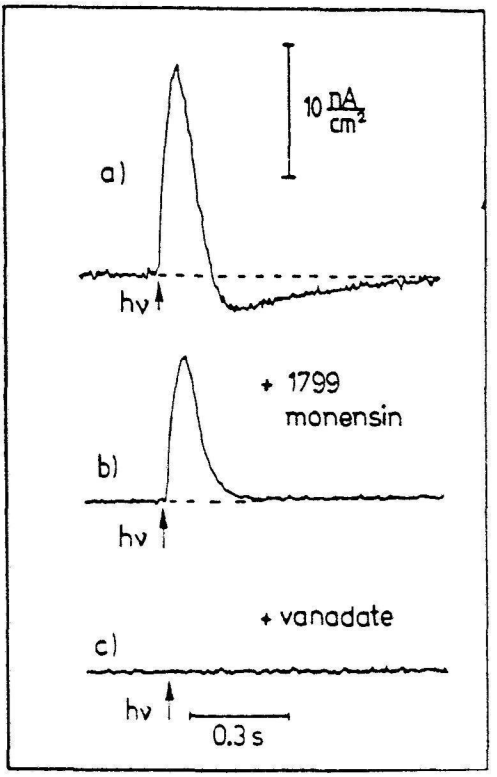

Fig. 2
Short-circuit current on the lipid bilayer system under different experimental conditions. The membrane bathing solution contained 3 mM $MgCl_2$, 50 mM imidazole pH 6.0 and 100 µM caged ATP. The light intensity was 3.7 W/cm², and the flash duration was 125 ms. The arrow indicates the opening of the shutter. Trace a: transient current after addition of 25 µl purified H,K-ATPase, 5 mg protein/ml. Trace b: pump current after addition of 10 µM monensin and 1 µM 1799. Trace c: blockade of the transient current by 20 µM vanadate.

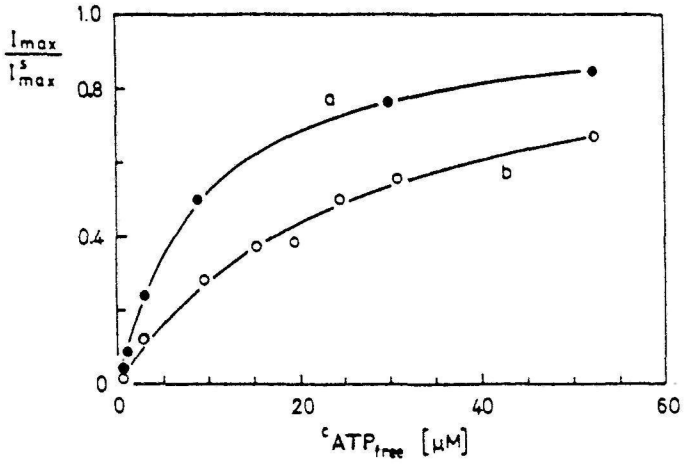

Fig. 3
ATP dependence of the peak current, measured by two different procedures. Electrolyte composition was the same as in Fig. 2. Trace a: The conversion rate of caged ATP was kept at 30%, variation of released ATP was obtained by addition of caged ATP. Trace b: Initial concentration of caged ATP was kept at 100 µM. The release of ATP from caged ATP was varied by changing the rate of photolysis. U.v. light was attenuated with u.v. filters. The peak current was normalized to $I_{max} = 1$ which is the saturating current response at cATP→∞. Trace a: $K_{0.5}$ = 9.4 µM. Trace b: $K_{0.5}$ = 26 µM.

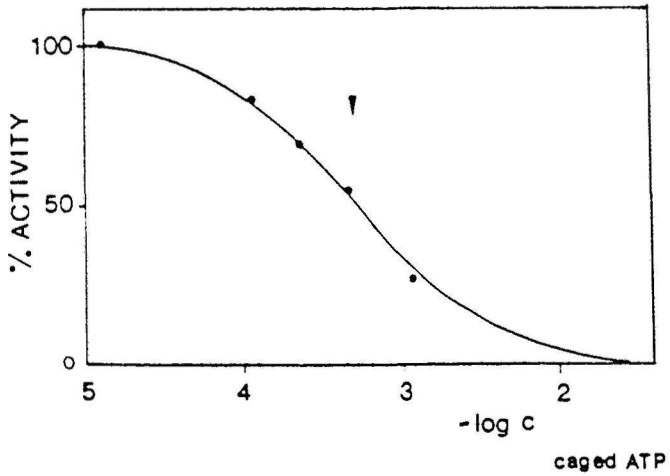

Fig. 4
Inhibition of enzymatic H,K-ATPase activity at pH 7.5 by the Tris salt of caged ATP at 37°C: Plot of % activity in dependence of the negative logarithm of caged ATP concentration. The solid line corresponds to a theoretical inhibition curve with an equilibrium constant of 5 x 10^{-4} M at 5 mM ATP. Further details are given in Materials and Methods.

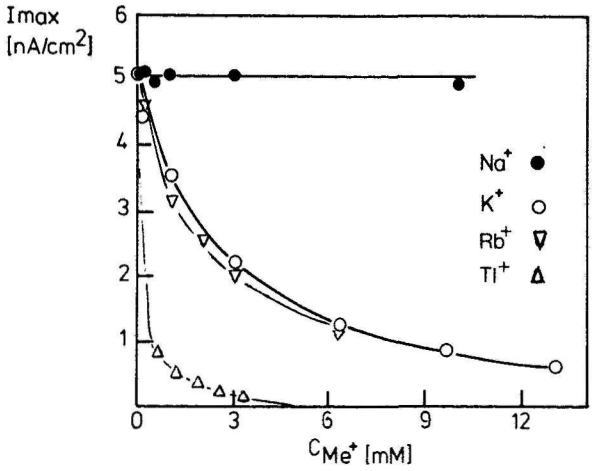

Fig. 5
Dependency of the peak current on monovalent cations. The cuvette contained 100 µM caged ATP, and the conversion rate was 30%. 125 µg purified membrane sheets containing H,K-ATPase were added to the membrane. The electrolyte composition was the same as in Fig. 2 except for the additional monovalent cations Na^+ (●) K^+ (o) Rb^+ (▽) and Tl^+ (△) at the indicated concentrations.

no effect on the signal, showing that the membrane fragments are adsorbed to the lipid film rather than integrated into it. In the latter situation, the oppositely-oriented fraction of membrane fragments should show the usual signal in response to ATP and its blockade by vanadate.

As shown in previous papers, involving the Na,K-ATPase, different control experiments were carried out to exclude possible artifacts by the photoreaction of caged ATP (Fendler et al., 1985, Nagel et al., 1987). In brief, the following controls also were made for the H,K-ATPase:

1.) The pure lipid bilayer did not show any effect after a u.v. flash of 125 ms and 3.7 W/cm^2 light intensity.

2.) The bilayer with adsorbed protein fragments showed no effect in the absence of caged ATP.

3.) The bilayer in presence of 50 μM caged ATP alone showed a small (10 pA/cm^2) photoeffect, which is due to the protonation of the lipid bilayer and the concomitant capacitive current. 1 H^+ per molecule caged ATP was released during the photoreaction. This current depended strongly on the buffer concentration and disappeared at concentrations of about 100 mM imidazole at pH 7.0 (Christensen et al., 1988).

4.) The addition of the ionophores 1799 and monensin did not cause further photoeffects.

5.) Prior addition of ATP (100 μM) in absence of K^+ to the lipid bilayer with adsorbed H,K-ATPase abolished the electrical response after an ATP-releasing flash because under these conditions all of the enzyme was already in the phosphorylated form.

Ion selectivity, ATP, ADP and P_i dependency and the inhibitory effect of caged ATP on the transient current

Fig. 3 shows the dependence of the transient current on the concentration of ATP. The two traces were obtained in different ways. In trace a, increasing amounts of caged ATP were added to the protein-containing side, where the conversion rate of ATP from caged ATP was kept constant at 30%. Trace b represents the ATP dependency when 100 μM caged ATP was added and the conversion rate increased by increasing light intensity. In the second of these two procedures the ratio of caged ATP to ATP was changed, so that an inhibitory effect of caged ATP, if present, should be detectable. As seen in Fig. 3, the two traces do not differ substantially so that an inhibitory effect of caged ATP on the enzyme appears neglegible. Depending on the experimental procedure, an apparent K_m for ATP of 9.4 μM (trace a) and 25 μM (trace b) was found. In parallel, the effect of caged ATP was studied in an enzymatic assay. The ATPase activity was measured at a fixed ATP concentration (5 mM) and increasing amount of caged ATP. An inhibition constant of the enzyme activity with an $I_{0.5}$ of 5×10^{-4} M was found, which is 100 times bigger than the $K_{0.5}$ for ATP (Fig. 4). This result indicates that the inhibition of the ATPase activity by caged ATP is neglegible for the H,K-ATPase. The inhibitory effect, however, should be taken in account for a kinetic analysis as shown for the Na,K-ATPase (Borlinghaus et al., 1987, Fendler et al., 1987).

The ion selectivity of the electrical signal with respect to monovalent cations is represented in Fig. 5. Sodium ions have no effect on the current response of the enzyme in the measured concentration range from 0 - 9 mM. K^+ and Rb^+ show a decrease of the peak current with an apparent inhibition constant of 3 mM at 30 μM released ATP. This effect is more pronounced for Tl^+ ions with an apparent inhibition constant of 100 μM.

The inhibition can partially be explained by a decrease in the affinity of the ATP-binding site for the substrate produced by cations like K^+, Rb^+ and Tl^+ (Schrijen et al., 1980). In the absence of K^+ and in presence of 30 μM ATP (from 100 μM caged ATP), saturating for phosphorylation, the peak current was measured (Fig. 6). Then 3 mM K^+ was added which reduced the peak amplitude to the half maximal value. Further release of ATP from caged ATP up to 120 μM restored the current response almost completely (Fig. 6). When, however, a similar experiment was carried out with 50 mM instead of 3 mM K^+, the peak current was abolished completely and could not be restored by concentrations of released ATP up to 200 μM (data not shown). This can be understood on the basis of the kinetics of the proton pump cycle. (see Discussion)

The H,K-ATPase is a Mg^{2+} dependent pump.

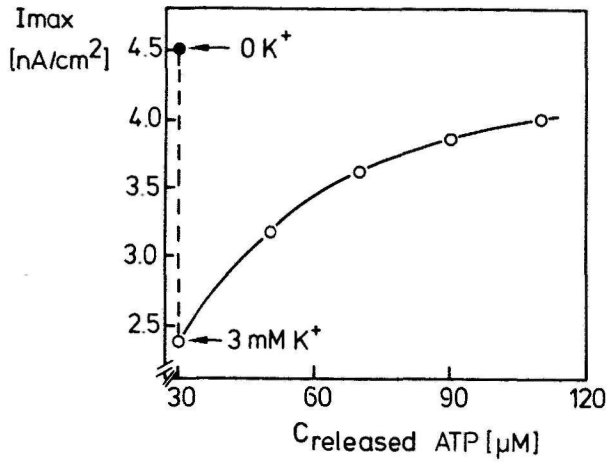

Fig. 6
Demonstration of the decrease in the affinity for ATP in presence of K^+. In the presence of 100 μM caged ATP, and a conversion rate 30%, and in the absence of K^+ the peak current was measured (●), addition of 3 mM K^+ reduced the peak current (o). Thereafter the peak current was measured after subsequent additions of caged ATP. The initial electrolyte composition was the same as in Fig. 2.

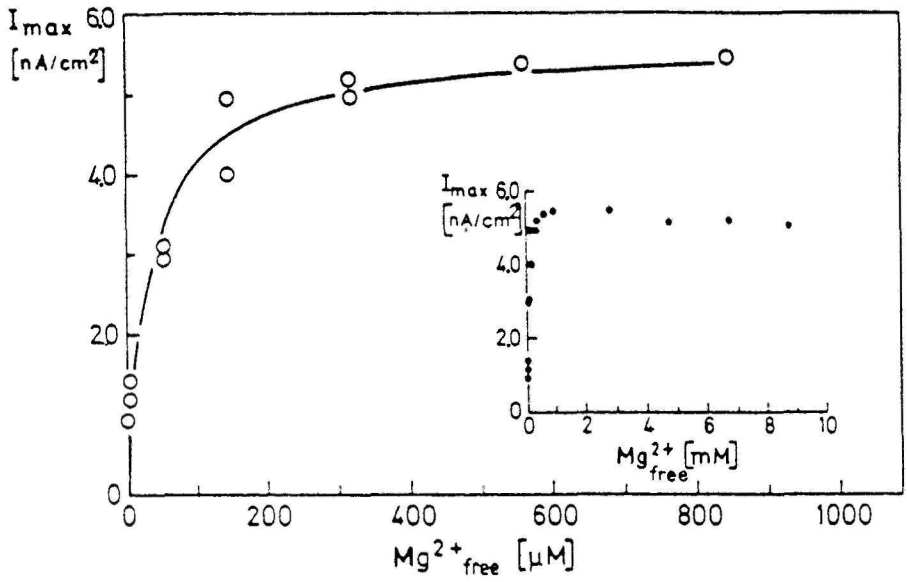

Fig. 7
Mg^{2+} dependency of the peak currents. Electrolyte composition: imidazole 50 mM, pH 6.0. After adsorption of the membrane fragments to the planar lipid bilayer 10 mM EDTA was added. Thereafter 100 μM caged ATP (conversion rate 30%) was added and the peak current was measured at increasing concentrations of free Mg^{2+}. A $K_{0.5}$ of 27 μM was obtained. The inset shows the dependency up to 10 mM Mg^{2+}.

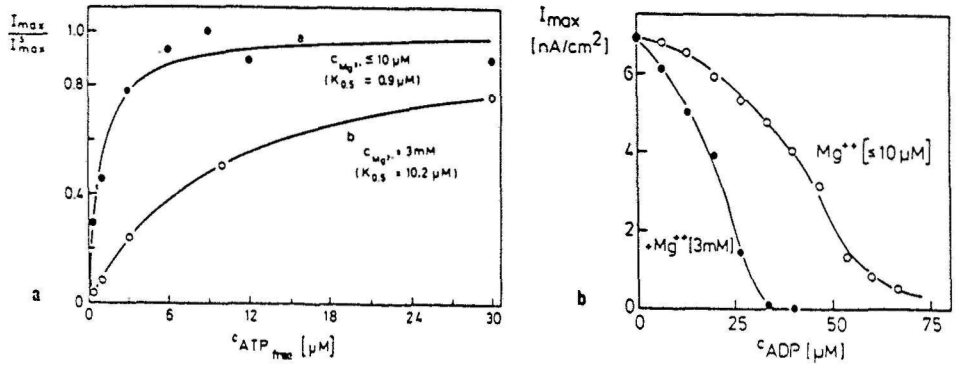

Fig. 8a *(left)*
ATP dependency of the peak current at different concentrations of free Mg^{2+}.
Trace a: 10 µM free Mg^{2+},
Trace b: 3 mM free Mg^{2+}. Other conditions as in Fig. 2.
The peak current was normalized to $I_{smax} = 1$, which is the saturating current response at cATP→∞. A $K_{0.5}$ of 0.9 µM (trace a) and 10.2 µM (trace b) was obtained.

Fig. 8b *(right)*
Decrease of the peak current by ADP. 100 µM caged ATP was used (conversion rate 30%). 3 mM $MgCl_2$, and 50 mM imidazole, were present throughout pH 6.0, the peak currents were measured at ADP concentrations as indicated (●). In absence of added Mg^{2+} the experiment was repeated (o). The latter trace (o) was normalized to the first one (●).

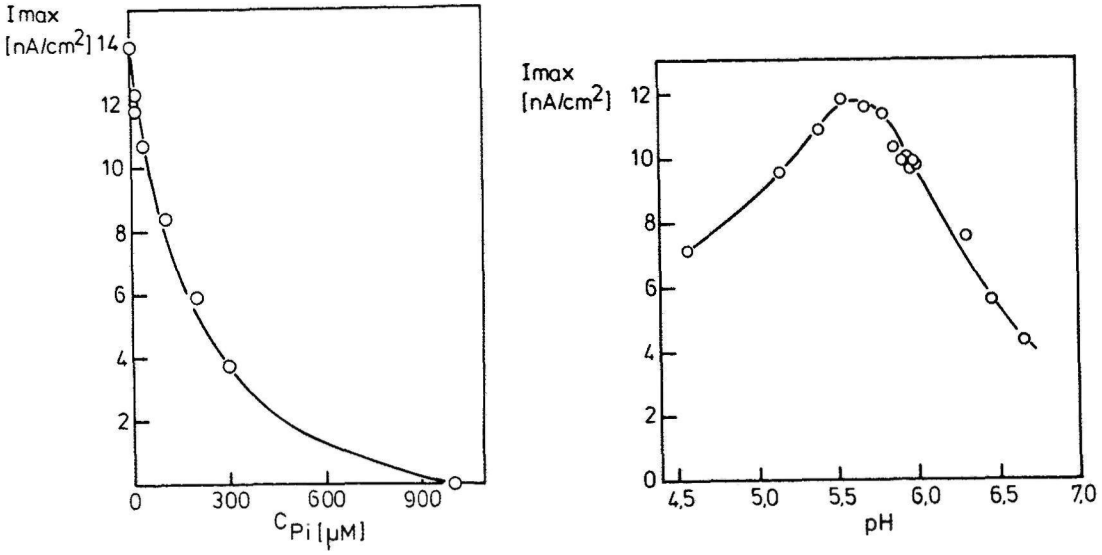

Fig. 9 *(left)*
Decrease of the peak current by inorganic phosphate (P_i). Using the conditions described in Fig.2, the peak current was measured in presence of added P_i at the indicated concentrations.

Fig. 10 *(right)*
pH dependence of the peak current in the presence of 100 µM caged ATP (conversion rate 30%). The data were obtained on the same membrane. The pH was varied in both compartments by addition of NaOH and H_2SO_4, respectively. Starting conditions: 50 mM imidazole, 3 mM Mg^{2+}, pH 5.99.

Therefore the removal of Mg^{2+} should abolish the transient current response. In absence of Mg^{2+} no current was obtained. The dependency of the transient current on the free Mg^{2+} concentration which was adjusted with EDTA is shown in Fig. 7. An apparent K_m for Mg^{2+} was found to be 27 μM. At high concentrations of free Mg^{2+}, a slight decrease of the current could be observed, which might be related to lower affinity for ATP (Fig. 8a). It is interesting that the affinity for ATP ($K_{0.5}$ = 0.9 μM) is considerably higher in presence of low Mg^{2+} (10 μM) than at a concentration of 3 mM which gives a $K_{0.5}$ of 10.2 μM (Fig. 8a). The different ATP affinities at different Mg^{2+} concentrations can be tentatively explained, by assuming that ATP binds first and then Mg^{2+} or vice versa, but that MgATP is not bound to the enzyme or that it has a lower affinity than ATP.

The electrical signal is inhibited by the addition of ADP. The inhibition is slightly dependent on the free Mg^{2+} concentration such that at high Mg^{2+} a higher affinity for ADP is found compared to low Mg^{2+} concentrations (Fig. 8b).

That the electrical signal represents a part of the pump cycle of the H,K-ATPase is further demonstrated by the inhibition of the signal by inorganic phosphate (P_i). Fig. 9 shows the decrease of the electrical current after successive additions of P_i. This inhibition may result from the ability of P_i to convert the enzyme into its phosphorylated state (E_2P). For further details see also Discussion, Fig. 12.

Finally, the pH dependency of the electrical signal was measured. A maximum for the current was obtained at pH 5.8 (Fig.10) which is in agreement with the pH optimum of 6.0 for the phosphorylation reaction (Stewart et al., 1981).

The effect of the H,K-ATPase specific inhibitor omeprazole on the ATP-induced transient current.

Omeprazole is a specific inhibitor of the gastric H,K-ATPase. Addition of omeprazole (10 μM) to the protein-containing compartment of the cuvette indeed abolished the peak current almost completely (Fig. 11). The lipid bilayer system offers the possibility of studying the mechanism of activation of the lipophilic compound. In the following experiments, a membrane bound mechanism for the activation (protonation) of the inhibitor was tested. The possibility of activation by acid groups of the enzyme which can react with omeprazole during the H^+ pumping phase of the reaction cycle was considered. Such acid groups seem to be necessary because protons are released into the lumen, where the pH is 1. The groups should be located close to the extracellular side, which faces in our experiments the planar lipid film. According to the sign of the current the protons are moved into the interfacial space. In the absence of omeprazole and K^+, repetitive measurements of the transient current gave reproducible signals after 5 min with an accuracy of 5-10%. Then 30 μM omeprazole was added to the protein-free side of the membrane and the pump was activated by a light-induced concentration jump of ATP from caged ATP. After 27 min incubation time with omeprazole, the current amplitude was reduced to 20 - 30% compared with the original value in the omeprazole-free medium. Further u.v. flashes releasing ATP increased the steepness of the time dependent inactivation curve drastically. Finally, after about 10 flashes, the current disappeared almost completely (Fig. 11). The repetition of the same experiment in presence of 3 mM K^+ did not show the increased inhibition. Addition of 1 mM DTT to the protein-containing compartment protected the enzyme partially from the inhibition by omeprazole (data not shown). Addition of monensin to both sides of the membrane in order to release transported protons from the interstitial space did not change the inhibition curve. These results suggest that a drop in the pH of the interstitial space is not the main reason for inactivation of the pump by omeprazole and that activation of the drug may occur at neutral pH.

Discussion

The pump cycle of the H,K-ATPase can be described like the cycle of the Na,K-ATPase by the Albers-Post scheme, shown in Fig 12. Many similarities between the two enzymes are apparent. One of the main differences, however, is that the Na,K-ATPase is an electrogenic pump, where the Na^+ transport contributes to the

electrogenicity (Fendler et al , 1985, Karlish et al , 1985, Gadsby et al., 1985, Borlinghaus et al , 1987, Nagel et al., 1987) The potassium transport in the sodium pump is electroneutral (Karlish et al , 1985, Goldschlegger, et al , 1987, Bahinski et al , 1988) In this paper, the transient current generated during phosphorylation of the electroneutral H,K-ATPase were studied. This was possible by separation of the H^+-dependent phosphorylation step from the K^+-dependent dephosphorylation step The results show that phosphorylation of the pump by ATP in the absence of K^+ produces a transient biphasic electrical response, suggesting that proton-translocation is electrogenic As a direct consequence of these experiments, the transport of potassium must be electrogenic, too, since no net stationary current was obtained, when H^+ and K^+ were both present

These experiments strongly support sequential transport of H^+ and K^+ ions and thus any model including the Albers-Post scheme, which is based on sequential transport. In principle, after a sudden release of ATP a transient electrical current either monophasic or biphasic should occur reflecting the H^+ and K^+ translocation which are oppositely directed Considering the data obtained from the kinetics of the enzymatic reactions it seems understandable that no current occurs in the presence of high potassium and high ATP At high potassium concentration the enzyme is in the E_2K or E_1K form with a low affinity for ATP The transition of E_2K $(E_1K)^{ATP}$ E_2K ATP (E_1K ATP) is slow (250 min^{-1}), whereas the subsequent transition from E_1 ATP---→E_1P + ADP is fast (4400-7900 min^{-1}) (Wallmark and Mårdh, 1979, Ljungström et al , 1984) A cycle starting with enzyme in the E_2K form will not give an electrical response, since a slow process (oppositely directed electrogenic or electroneutral) precedes the fast H^+-dependent electrogenic step The demonstration of the proton transporting step in the absence or at low concentrations of potassium shows that the reaction under these conditions always starts in the E_1H form (Fig 12)

Since only counter-transported ions like K^+, Rb^+ and Tl^+ in contrast to Na^+ have an effect on the electrical response and no stationary currents can be observed, one may conclude that the electrical signal represents proton transport and not a dipole movement during a conformational change within the protein The ion specificity of the electrical current shown in Fig 5 agrees well with data obtained by Ray and Forte, (1976), and Sachs et al , (1976), who showed that the reduction of the phosphorylated intermediate decreases with the same specificity for Na^+, K^+, Rb^+, and Tl^+

The effect of Mg^{2+}, ATP, ADP, and P_i

Mg^{2+} influences the activation of the transient current with respect to the affinity of ATP (Fig 8a) The high affinity for ATP at low concentrations of Mg^{2+} suggests that the affinity for ATP is higher than for MgATP Since the enzyme contains tightly bound Mg^{2+} (50 nmol/mg protein) for steric reasons it is expected to bind ATP better to the enzyme than preformed MgATP Similar results were obtained in ATP dependent proton transport studies on membrane vesicles (Ljungström & Mårdh, 1985). These results agree within a factor of 3 with the electrical measurements presented above The affinity of ADP shows the opposite Mg^{2+} dependence to that of ATP at high and low concentrations of Mg^{2+} (Fig 8b) In the presence of high Mg^{2+} (3 mM) the high affinity for ADP can be explained by a Mg^{2+} dependent binding to the E_1P form Then the inhibition of the current by ADP can be explained by two mechanisms a) competition of ADP with the ATP binding site E_1H^+ or b) binding of ADP to the E_1P form which drives the back reaction

The inhibition of the electrical current by inorganic phosphate P_i reflects the formation of E_2P The $K_{0.5}$ of 150 μM is in good agreement with a $K_{0.5}$ of 60 μM obtained from phosphorylation experiments (Jackson and Saccomani, 1984)

The effect of omeprazole

Omeprazole is a specific inhibitor of the gastric H,K-ATPase At neutral pH omeprazole has hydrophobic properties and is membrane permeable so that it can penetrate into the ATPase rich vesicles The inhibitory activity under these circumstances is low Activation of the pump by ATP acidifies the intravesicular medium Thereby omeprazole is decomposed at

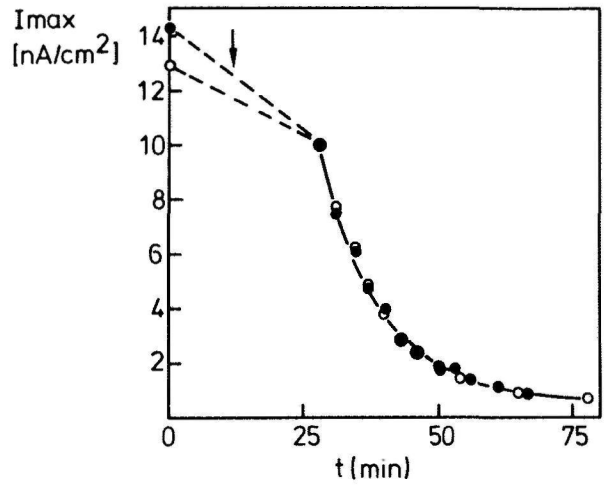

Fig. 11
Inhibition of the peak current in presence of 30 µM omeprazole, without (o) and with (•) the H^+, Na^+ exchanging carrier monensin (10 µM) 10 mM NaCl was present. The arrow indicates the addition of omeprazole. The curves are normalized to each other. Other conditions as in Fig. 2.

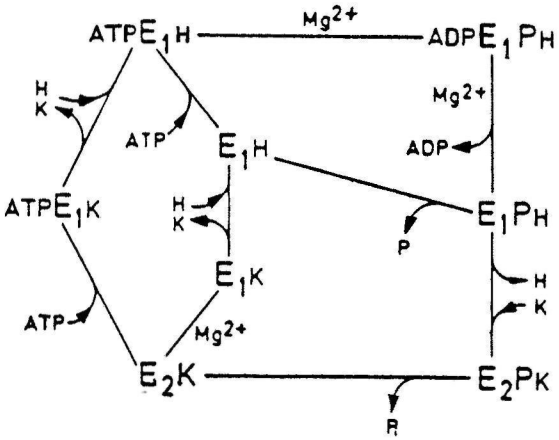

Fig. 12
Representation of the reaction cycle of the gastric H,K-ATPase in a modified Albers-Post scheme according to Stewart et al., 1981, Sachs et al., 1982.

pH 4 to a hydrophilic compound (pK_a=4) which is trapped within the vesicles In its decomposed form omeprazole develops full inhibitory activity as an SH reagent (Fellenius et al 1981, Larsson et al , 1983, Wallmark et al , 1984, Im et al , 1984, Lindberg et al, 1986) Results of the present study suggest that inactivation of the H,K-ATPase is not necessarily dependent on being in a low pH aqueous compartment The experiment shown in Fig 11 raise the question if omeprazole in addition to its efficacy at low pH can be activated by the pump itself In an almost perfectly buffered system the inactivation increases the more the pump is inactivated The addition of the H^+/Na^+ exchanging carrier monensin which removes a possible H^+ gradient from the interstitial space did not change the inhibition curve Control experiments with the light driven proton pump bacteriorhodopsin showed an inhibition of the pump by acidification of the interstitial space between the adsorbed purple membranes and the underlying lipid film This effect could completely be removed by the addition of the ionophoric system monensin plus the protonophore 1799 (data not shown) As shown in Fig 11 the addition of the same ionophoric system had no influence on the inactivation by omeprazole, so that in this particular experiment activation of the drug by acidification of the interstitial space might be excluded In other words a blockade of the H,K-ATPase by omeprazole close to neutral pH (pH = 6 4) is possible and can be explained as follows In the inactive hydrophobic form omeprazole is expected to be located in the membrane During the H^+ transport step of the H,K-ATPase, possibly extremely acidic groups may be exposed to the membrane interface, which are not accessible to the well buffered aqueous bulk phase This is reasonable because the pump releases protons into a medium of pH 1 These groups might initiate the H^+-dependent decomposition of omeprazole, so that the inhibition occurs after activation of the pump during the formation of E_1P or E_2P The experiments were carried out at zero K^+ which means that the phosphorylated form remains stable for a longer period of time than under physiological conditions so that an increased inhibition is probable This is supported by experiments where in the presence of 3 mM K^+ an increased inhibition at pH 6 4 was not observed, where dephosphorylation decreases the concentration of the phosphorylated intermediates

Acknowledgements

The authors wish to thank A Hüby and A Ifftner for excellent technical assistance and Dr J Froehlich for valuable comments and critical reading of the manuscript This study was supported partially by the Deutsche Forschungsgemeinschaft (SFB 169) and by the Netherlands Organization for Scientific Research (N W O), through the Netherlands Biophysics Foundation One of us, H T W M van der Hijden, was a recipient of a Max-Planck fellowship

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Phosphorylation of $(H^+ + K^+)$-ATPase by inorganic Phosphate. The role of K^+ and SCH 28080.

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Submitted for publication J. Biol. Chem.

Abbreviations: P_i inorganic phosphate

$EP_{(P_i)}$ and $EP_{(ATP)}$ represent the phosphointermediate formed by phosphorylation with inorganic phosphate and ATP, respectively

SCH 28080 {2-methyl-8-[phenyl methoxy] imidazo- (1-2-a) pyrine-3-acetonitrile}

Keywords: H,K-ATPase
P_i phosphorylation
dephosphorylation
SCH 28080

Phosphorylation of H,K-ATPase by inorganic phosphate. The role of K^+ and SCH 28080.

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SUMMARY

The effects of K^+ on the phosphorylation of H,K-ATPase with inorganic phosphate were studied using H,K-ATPase purified from porcine gastric mucosa. The phosphoenzyme formed by phosphorylation with P_i was identical with the phosphoenzyme formed with ATP. The maximal phosphorylation level obtained with P_i was equal to that obtained with ATP.

The P_i phosphorylation reaction of H,K-ATPase was like that of Na,K-ATPase a relatively slow reaction. The rates of phosphorylation and dephosphorylation were increased by low concentrations of K^+ which resulted in nearly no effect on the phosphorylation level. A decrease of the steady state phosphorylation level was caused by higher concentrations of K^+ in a noncompetitive manner, whereas no further increase of the dephosphorylation rate was observed. The decreasing effect was caused by a slow binding of K^+ to the enzyme.

All above mentioned K^+ effects were abolished by SCH 28080. Additionaly, SCH 28080 caused a twofold increase in the affinity of H,K-ATPase for P_i. A model for the reaction cycle of H,K-ATPase fitting the data is postulated.

INTRODUCTION

H,K-ATPase is a member of the P-type transport ATPases, a class of membrane bound ion-transporting ATPases that accept the terminal phosphate group of ATP to form a phosphorylated intermediate and then donate the phosphate group to water during the reaction sequence (1) Walderhaug et al (2) showed that during the phosphorylation reaction of H,K-ATPase the gamma phosphate group of ATP is bound to an aspartyl residue of the enzyme. The dephosphorylation rate of the phosphorylated intermediate is stimulated by K^+ (3)

It has been shown that H,K-ATPase (4), like the other P-type ATPases Na,K-ATPase and Ca-ATPase (5,6), can be phosphorylated with P_i as well The extensive studies on P_i phosphorylation of Na,K-ATPase have given clues to the mechanism of this enzyme A study towards the P_i phosphorylation of H,K-ATPase was also expected to contribute to further insight in its reaction mechanism

Hitherto not much is known about the P_i phosphorylation of H,K-ATPase This paper presents studies on the P_i phosphorylation of this enzyme Firstly the characteristics of the P_i phosphorylation reaction are determined Secondly the phosphoenzymes formed with P_i and ATP are compared Thirdly the effects of K^+ on the

P_i phosphorylation are studied And lastly the effects of the specific H,K-ATPase inhibitor SCH 28080 on the P_i phosphorylation and on the effect of K^+ on this reaction are determined

MATERIALS AND METHODS

Enzyme preparation

Isolation of H,K-ATPase containing membrane sheets was carried out according to the procedure reported by Skrabanja et al (7) The isolated preparation was stored at - 20°C in 0 15 M sucrose The specific activity of the enzyme preparations ranged from 50 to 100 µmol ATP hydrolysed per hour per mg protein at 37°C The protein determination was done according to Lowry et al (8) using bovine serum albumin as standard

ATPase activity assay

K^+ stimulated ATPase activity was determined according to Schrijen et al (9) Gastric H,K-ATPase was incubated at 37°C in the presence of 30 mM imidazole/HCl (pH 7 0), 5 mM Mg^{2+}, 0 1 mM ouabain, 5 mM ATP and 20 mM K^+ or 20 mM choline chloride for 20 min The difference between the P_i production with and without K^+ present was taken as the H,K-ATPase activity Stopping of the reaction and further treatment of the samples were carried out as described before (10)

Phosphorylation with ATP

Phosphorylation was carried out at 20°C for various periods of time with 5 µM [gamma ^{32}P]ATP (The Radiochemical Center, Amersham UK, specific radioactivity was adjusted with nonradioactive ATP to 90 to 150 Ci/mol) in a buffer medium (100 µl) containing 50 mM imidazole/HCl (pH 6 0), 1 mM Mg^{2+} and 0 1 to 0 2 mg/ml H K-ATPase protein The reaction was started by rapid mixing of the medium containing the enzyme (90 µl) with 10 µl [gamma ^{32}P]ATP and was stopped by addition of 3 ml 5% (w/v) trichloroacetic acid, containing 100 mM phosphoric acid The denatured phosphoprotein was filtered on a 1 2 µm pore width selectron filter (Schleicher and Schüll, Dassel, FRG), which was then three times washed with stopping solution Incorporated ^{32}P was determined by liquid scintilation counting For blank values the stopping solution was mixed with the enzyme prior to addition of [gamma ^{32}P]ATP

Phosphorylation with inorganic phosphate

Phosphorylation with $^{32}P_i$ (The Radiochemical Center, Amersham UK, specific radioactivity was ajusted with nonradioactive P_i to 50 to 200 Ci/mol) was carried out at 20°C for various periods of time in a medium containing 50 mM imidazole/HCl (pH 6 0), 1 mM Mg^{2+} and 0 15 to 0 20 mg/ml H,K-ATPase protein The reaction was started and stopped as described for phosphorylation with [gamma ^{32}P]ATP The denatured phosphoprotein was collected, washed and the incorporated ^{32}P was determined For blank values the stopping solution was mixed with the enzyme prior to addition of ^{32}P

Dephosphorylation of the phosphoenzyme formed with P and ATP

The dephosphorylation assay was carried out at 20°C after phosphorylation of the enzyme with either $^{32}P_i$ or [gamma ^{32}P]ATP To 100 µl of the phosphorylation medium 900 µl dephosphorylation medium, containing 50 mM imidazole/HCl (pH 6 0), 1 mM Mg^{2+}, 0 1 mM nonradioactive ATP and 1 mM nonradioactive P_i was added

Nonradioactive ATP (0 1 mM) was added in excess to prevent incorporation of ^{32}P (from [gamma ^{32}P]ATP) Nonradioactive P_i was added in excess to prevent the incorporation of ^{32}P (from $^{32}P_i$) The dephosphorylation reaction was stopped as described for phosphorylation with [gamma ^{32}P]ATP The denatured phosphoenzyme was collected, washed and the incorporated ^{32}P

was determined by liquid scintilation analysis.

Hydroxylamine assay

The sensitivity of the phospho-intermediates formed by P_i and ATP (as described above) to hydroxylamine was performed as described by Schuurmans Stekhoven et al (11).

Determination of the K^+ concentration present in the phosphorylation assay mixture

The K^+ concentration present in the phosphorylation mixture, in absence of added K^+, was determined by flame photometry using an Eppendorf FCM 6343 apparatus.

Materials

ATP was purchased from Boehringer, Mannheim, F.R.G.; [gamma ^{32}P]ATP and $^{32}P_i$ were obtained from Amersham, Buckinghamshire, U.K.; SCH 28080 {2-methyl-8-[phenyl methoxy] imidazo- (1-2-a) pyrine-3-acetonitrile} and omeprazole were kindly provided by Dr. B. Wallmark, Hässle, Sweden; vanadate was purchased from Riedel de Haën, AG., Seelze, Hannover F.R.G.

RESULTS

Properties of P_i phosphorylation of H,K-ATPase

The phosphorylation reaction of H,K-ATPase by P_i at 20°C in the absence of added K^+ is a rather slow reaction with a pseudo first-order rate constant (k_{app}) of 0.09 s^{-1}. The rate of phosphorylation is not significantly affected by pH values between 6 and 8. A maximal phosphorylation level was reached at a pH between 6 and 7.

The K_m for P_i, in the presence of 50 mM imidazole/HCl (pH 6.0) at a saturating Mg^{2+} concentration of 1 mM, is 47 ± 8 µM (Table I). These observations are in accordance with those of Jackson and Saccomani (12).

Comparison of the phosphoenzymes formed by phosphorylation with P_i ($EP_{(P_i)}$) and ATP ($EP_{(ATP)}$)

Vanadate, a known inhibitor of P-type transport ATPases (1,13,14), inhibits the reaction cycle of these ATPases by occupation of the ATP phosphorylation site (15). This inhibitor also inhibited the P_i phosphorylation in a competitive manner (Figure 1) with a K_i of 0.3 µM which is in accordance with observations of Faller et al (14).

Omeprazole, a specific inhibitor of H,K-ATPase (16,17,18) inhibited the phosphorylation reactions with both P_i and ATP in a similar way with an IC_{50} of 180 and 30 µM respectively (not shown).

Both phosphoenzymes formed by either P_i or ATP phosphorylation were hydroxylamine-sensitive, showing that the two phosphointermediates were mixed anhydrides. This suggests that both P_i and the phosphate group of ATP bind to an aspartate residue.

To examine whether $EP_{(Pi)}$ and $EP_{(ATP)}$ are indeed the same phosphoenzymes the maximal phosphorylation level reached with either P_i (500 µM, 10 times the K_m) or ATP (at a saturating concentration of 5 µM) was determined for 6 different H,K-ATPase preparations. The ratio of the steady state phosphorylation levels obtained with P_i and ATP was 1.03 ± 0.04 (mean ± SEM, n=6).

To establish that phosphorylation by P_i forms the same phosphoenzyme as phosphorylation by ATP a competition experiment as shown in Figure 2 was carried out. H,K-ATPase was phosphorylated for 60 s with increasing concentrations of nonradioactive P_i in order to obtain a certain submaximal level of phosphorylated intermediate. Subsequently radioactive labeled ATP at a saturating concentration was added and incubated for 4 s in order to phosphorylate the remaining unphosphorylated enzyme. In a parallel experiment the enzyme was first phosphorylated by

	Control	SCH 28080	ratio
n	6	4	4
K_m	47 ± 8 μM	17 ± 3 μM	0 41 ± 0 04*
EP_{max}	604 ± 63 pmol/mg	689 ± 91 pmol/mg	1 12 ± 0 04*

Table I The effects of SCH 28080 on the K_m for P_i and the EP_{max}
H,K ATPase (200 μg/ml) from several different preparations was phosphorylated with a range of P_i concentrations in the presence of 1 mM Mg^{2+}, 50 mM imidazole (pH 6 0) with and without 0 1 mM SCH 28080 The reaction was stopped after 60 s and the level of the phosphorylated intermediate (EP) was determined From these values the K_m for P_i and the maximal phosphorylation level (EP_{max}) were obtained by Scatchard analysis The ratios of the K_m and of the EP_{max} in the presence and absence of SCH 28080 were determined per experiment These ratios were averaged and given as mean values ± standard error of the mean (* $P < 0\ 05$)

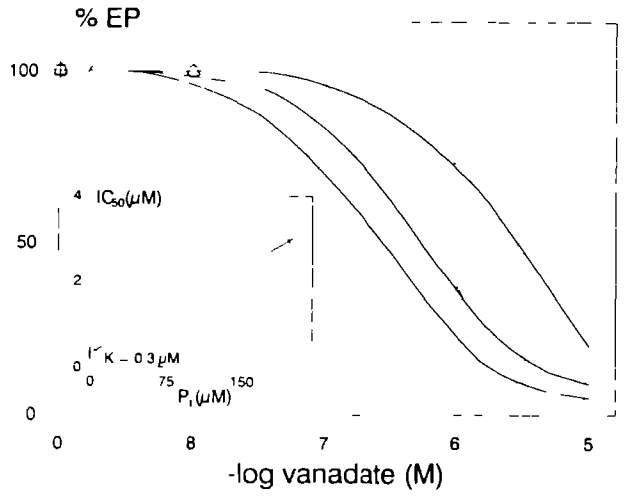

Figure 1 ***The inhibitory effect of vanadate on P_i phosphorylation***
H K-ATPase (150 μg/ml) was incubated in the presence of 1 mM Mg^{2+}, 50 mM imidazole/HCl (pH 6 0) and varying concentrations of vanadate Subsequently $^{32}P_i$ was added to a final concentration of 2 (□), 20 (△) or 200 μM (○) The reaction was stopped after 60 s and the level of phosphorylated intermediate (EP) was determined and expressed as percentage of the EP value in the absence of vanadate
Inset The IC_{50} values obtained from these experiments are plotted against the P_i concentration

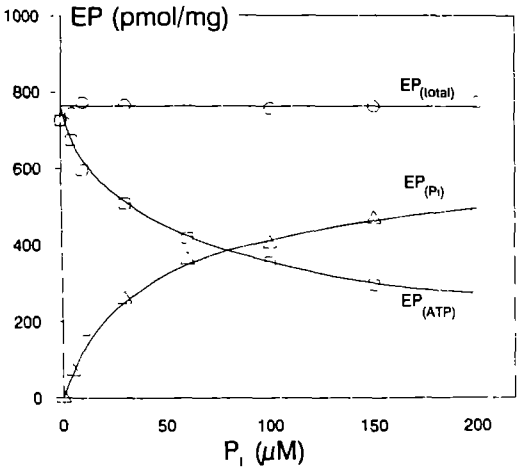

Figure 2. ***Exclusion of ATP phosphorylation by P_i phosphorylation***
H,K-ATPase (150 μg/ml) was preincubated for 60 s in presence of 1 mM Mg^{2+}, 50 mM imidazole/HCl (pH 6.0) with varying concentrations of $^{32}P_i$ (Δ) or P_i (□). Subsequently ATP (Δ) or [gamma ^{32}P] ATP (□) was added to a final concentration of 5 μM. The reaction was stopped 4 s after addition of ATP and the level of labeled phosphorylated intermediate ($EP_{(ATP)}$ or $EP_{(Pi)}$) was determined.
$EP_{(total)}$ (○) is the sum of $EP_{(Pi)}$ and $EP_{(ATP)}$.

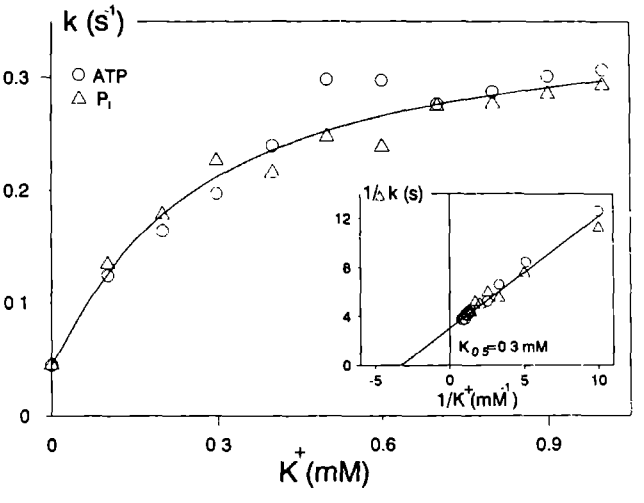

Figure 3. ***K^+ dependence of the dephosphorylation rate of EP formed by either P_i or ATP phosphorylation***
H,K-ATPase (150 μg/ml) was preincubated in the presence of 1 mM Mg^{2+}, 25 mM imidazole/HCl (pH 6 0) and 60 μM $^{32}P_i$ (○) or 5 μM [gamma ^{32}P] ATP (Δ) during 60 and 10 s respectively. Subsequently dephosphorylation medium, containing unlabeled ATP (0.1 mM) and P_i (1 mM) instead of the labeled compounds and the indicated K^+ concentrations, was added. The reaction was stopped after 3 s and the level of phosphorylated intermediate (EP) was determined. The rate constants were calculated asuming first order kinetics. In control experiments with K^+ concentrations first order kinetics were indeed measured during the first 10 s.
Inset: the $K_{0.5}$ value of 0.3 mM was obtained by Lineweaver Burk analysis.

labeled P followed by addition of non radioactive ATP under otherwise identical conditions Fig 2 shows that the sum of the labeled phosphorylated intermediates in the two experiments was constant at each P concentration indicating that P_i and ATP exclude each other from phosphorylation This mutual exclusion confirms that the $EP_{(Pi)}$ is the same phosphoenzyme as $EP_{(ATP)}$ with respect to the phosphate binding site

K^+ effects on the dephosphorylation rate of EP and on the P phosphorylation rate and level

Both 0 1 mM ATP and 1 mM P_i were added to the dephosphorylation medium in order to prevent further incorporation of labeled substrate in the enzyme Addition of ATP however influences the dephosphorylation rate as described by Helmich-de Jong et al (19) In order to obtain comparable results with both substrates, the same dephosphorylation medium was used for the dephosphorylation of the phosphointermediates formed by either ^{32}P or [gamma ^{32}P]ATP

In accordance with earlier results of Wallmark and Mårdh (3) the dephosphorylation reaction of $EP_{(ATP)}$ was stimulated by K^+ The pattern of stimulation of the dephosphorylation reaction of $EP_{(P_i)}$ is similar to that of $EP_{(ATP)}$ (Figure 3) The $K_{0.5}$ for K^+ with respect to the increase of the dephosphorylation reaction rate is 0 3 mM for both phosphointermediates

In contrast to ATP phosphorylation (3) the rate of the P_i phosphorylation reaction was also increased by K^+ The k for the phosphorylation reaction was increased by K^+ with a $K_{0.5}$ of 0 2 mM (Figure 4) In these calculations a correction has been made for the K^+ concentration of 19 µM (mainly originating from K^+ bound to the enzyme) present in the phosphorylation assay mixture in the absence of added K^+ The k for the phosphorylation reaction in absence of K^+ (0 06 s^{-1}) was obtained by extrapolation (Figure 4) The steady state phosphorylation level was hardly affected by K^+ concentrations up to 0 3 mM (not shown) A further increase of the K^+ concentration reduced the steady state phosphorylation level with a $K_{0.5}$ of 2 4 mM (Figure 5)

The time dependence of the formation of the phosphointermediate in the presence of K^+ is rather complex By simultaneous addition of K^+ (30 mM) and P_i (60 µM) a transient high phosphorylation level was reached within 3 s (Figure 6) The transient was the result of the above described effect of K^+ on the P_i phosphorylation rate and was followed by a slow decrease of the phosphorylation level to a lower steady state value in two min Preincubation of the enzyme with 30 mM K^+ shows that the latter effect was due to a slow binding of K^+, since no transient was observed and the same steady state phosphorylation level as that in the former experiment was reached within 3 s (Figure 6) The apparent decrease in K^+-sensitivity, as observed on figure 6 compared to figure 5, is due to inhibitory effects of high (above 10 mM) choline chloride concentrations present in the control experiment

The effect of K^+ on the K_m for P_i and on the maximal phosphorylation level is given in Figure 5 Since only the maximal phosphorylation level and not the K_m for P_i is influenced by K^+ the inhibitory effect of K^+ is noncompetitive

SCH 28080 effects on the phosphorylation and dephosphorylation reactions

The effects of the specific H,K-ATPase inhibitor SCH 28080 on the phosphorylation and the dephosphorylation reaction were studied

SCH 28080 inhibited the H,K-ATPase hydrolytic activity at pH 7 0 with an IC_{50} of 0 8 µM (cf ref 20 0 15 µM at pH 6 5, 1 5 µM at pH 7 5) and the phosphorylation reaction with ATP with an IC_{50} of 0 3 µM (cf ref 20 0 3 µM at pH 6 0 and 7 4)

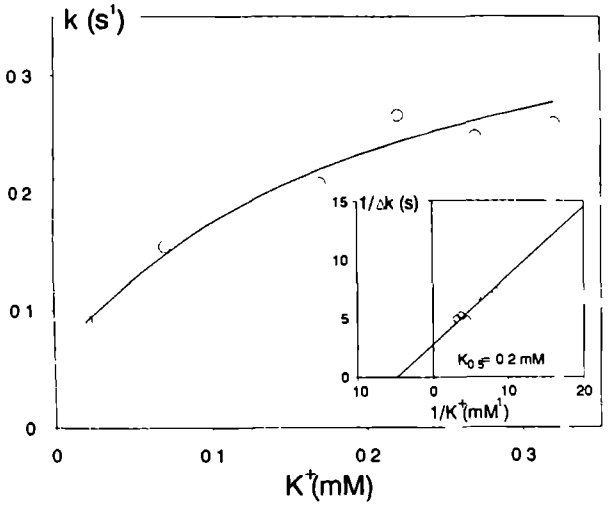

Figure 4. ***K⁺ dependence of the rate of formation of the phosphointermediate by P_i***

H K-ATPase (200 µg/ml) was preincubated in the presence of 1 mM Mg^{2+}, 50 mM imidazole/HCl (pH 6 0) and varying concentrations of K^+ Choline chloride was added to maintain constant salt concentrations of 0 3 mM Subsequently $^{32}P_i$ was added to a final concentration of 60 µM The reaction was stopped after various time intervals (3, 5, 7, 9 and 60 s) and the level of the phosphorylated intermediate (EP) was determined

The rate constants of these reactions were determined as the slope of the plot of $\ln(EP_{max}/EP_{max}$ EP) and time (EP_{max}=EP at 60 s) The rate constant as a function of the K^+ concentration is shown The assay mixture in absence of added K^+, including the enzyme, contained 19 µM K^+ The k value for 0 mM K^+ (k_0) was determined by extrapolation

Inset double reciprocal plot of k and the K^+ concentrations k was obtained after subtraction of the k_0 from the observed k

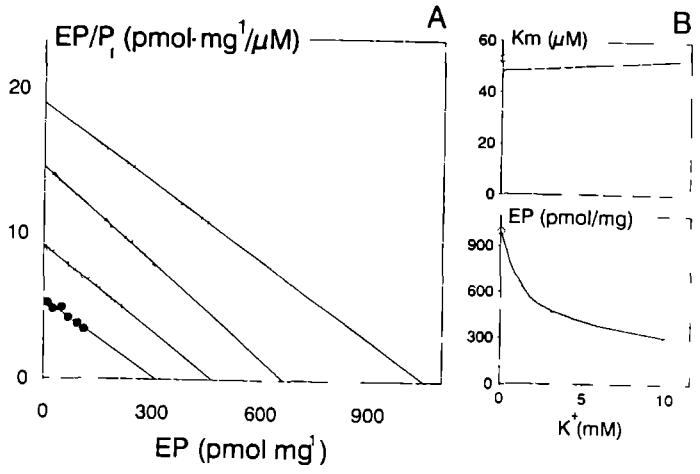

Figure 5 ***Effect of K^+ on the K_m for P_i and the maximal phosphorylation level of P_i phosphorylation***

A H K ATPase (150 µg/ml) was incubated in the presence of 1 mM Mg^{2+}, 50 mM imidazole/HCl (pH 6 0) with 0 (○), 1 (△), 3 (□) and 10 mM K^+ (●) for 30 min Choline chloride was added to maintain constant ionic strength Subsequently varying $^{32}P_i$ concentrations were added After 60 s the reaction was stopped and the level of phosphorylated intermediate (EP) was determined

B The values of K_m and EP_{max}, calculated from Figure 5A are given as function of K^+

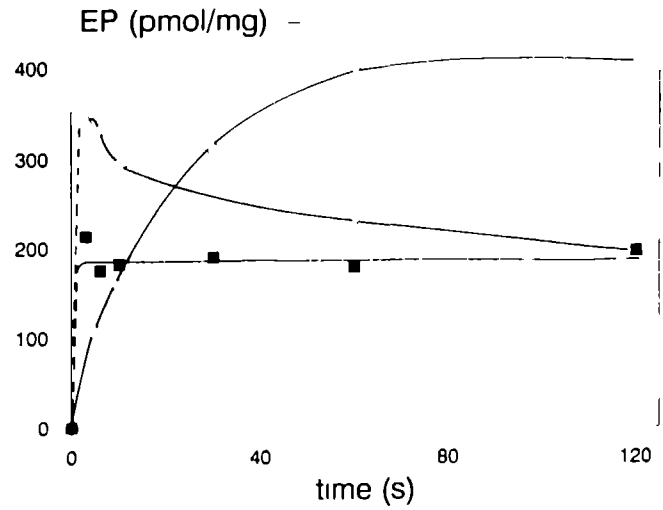

Figure 6. ***Time course for P_i phosphorylation in the presence and absence of 30 mM K^+***
H K-ATPase (200 µg/ml) was preincubated in the presence of 1 mM Mg^{2+}, 50 mM imidazole/HCl (pH 6 0), with (closed symbols) or without (open symbols) 30 mM K^+ for 30 min Subsequently $^{32}P_i$ (to a final concentration of 60 µM) and 0 (■), 30 mM K^+ (Δ) or 30 mM choline chloride (○) were added At the times indicated the reaction was stopped and the level of phosphorylated intermediate (EP) was determined

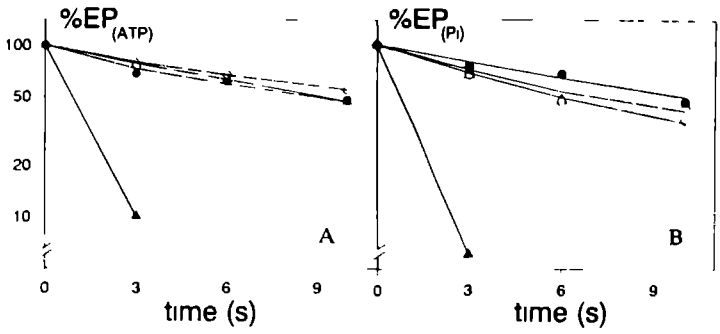

Figure 7. ***Effect of SCH 28080 on the K^+ stimulated dephosphorylation rate of $EP_{(Pi)}$ and $EP_{(ATP)}$***
A H K-ATPase (150 µg/ml) was phosphorylated in the presence of 1 mM Mg^{2+} and 50 mM imidazole/HCl (pH 6 0) with 5µM [gamma ^{32}P] ATP during 10 s Subsequently SCH 28080 was added to a final concentration of 0 (open symbols) or 10 µM (closed symbols) Dephosphorylation medium containing 10 mM choline chloride (circles) or 10 mM K^+ (triangles) was added 10 s after addition of SCH 28080 At the times indicated the reaction was stopped and the level of phosphorylated intermediate $EP_{(ATP)}$ was measured
B H K ATPase (150 µg/ml) was preincubated in the presence of 1 mM Mg^{2+} 50 mM imidazole/HCl (pH 6 0) 60 µM $^{32}P_i$ with (open symbols) or without (closed symbols) 0 1 mM SCH 28080 during 120 s Subsequently dephosphorylation medium containing 10 mM choline chloride (circles) or 10 mM K^+ (triangles) was added The reaction was stopped after the indicated time intervals and the level of phosphorylated intermediate $EP_{(Pi)}$ was determined

The effect of SCH 28080 on the phosphorylation with P_i, however, was opposite to that on the phosphorylation with ATP at suboptimal P_i concentrations the steady state phosphorylation level was markedly increased by increasing SCH 28080 concentrations Table I shows that this increase is mainly due to an increase in the affinity of the SCH 28080 bound enzyme for P A slight but significant increase in the maximal phosphorylation level of 12 ± 4 % (Table I) was also observed

Since it is suggested that SCH 28080 binds at the K^+ binding site (21) the inhibitor was expected to have an effect on the K^+ stimulated dephosphorylation reaction of both $EP_{(Pi)}$ and $EP_{(ATP)}$ This stimulation was in both cases totaly abolished by 0 1 mM SCH 28080 (Figure 7) No significant effect of SCH 28080 was found on the (basal) dephosphorylation rate in the absence of K^+

In the presence of 0 1 mM SCH 28080 the effects of K^+ on the steady state phosphorylation level and the phosphorylation rate were also abolished

As is shown in Figure 8 the phosphorylation rate in the presence of 0 1 mM SCH 28080 was no longer affected by 10 mM K^+ The rate constants (0 06 s^{-1}) were equal to those in the absence of K^+ and SCH 28080

SCH 28080 (0 1 mM) also abolished the reduction of the phosphorylation level by K^+ This occurred when K^+ was added either prior to SCH 28080 (Figure 9) or together with the substrate (Figure 8)

DISCUSSION

Comparison of the P_i and ATP phosphorylation reactions

The P_i phosphorylation of Na,K ATPase is a slow reaction (6) Since Na,K-ATPase and H K ATPase show numerous kinetic and structural similarities it could be expected that the P phosphorylation of H,K-ATPase is also a slow reaction as was indeed observed

K^+ stimulated ATPase activity and ATP phosphorylation of H,K-ATPase are both inhibited by vanadate and omeprazole (14,17,22). Vanadate also inhibits the P_i phosphorylation of Na,K-ATPase (23) This paper shows that vanadate also inhibits the P_i phosphorylation of H,K-ATPase in a competitive manner This is in accordance with suggestions made by Faller and Elgavish (15) Omeprazole also inhibited the P_i phosphorylation of H,K-ATPase

Beside the above mentioned similarities between the P_i and ATP phosphorylation reactions the hydroxylamine sensitivity of the phosphoenzymes formed by P_i and ATP was identical, which reflects the presence of a mixed anhydride of a carboxyl group in the enzyme and phosphate Since the rate of hydrolysis of this anhydride was the same for both $EP_{(Pi)}$ and $EP_{(ATP)}$ it is likely that the phosphointermediates are chemically identical As is shown by Walderhaug et al (2) phosphorylation with ATP yields a mixed anhydride of an aspartyl residue of the enzyme and the gamma phosphate of ATP Thus it is very likely that the phosphate of the phosphointermediate formed by P_i phosphorylation is also bound to an aspartyl residue

Further evidence for the identity of both phosphointermediates is provided by the fact that the phosphoenzyme formed by P_i can not be phosphorylated anymore by ATP Moreover, the maximal steady state phosphorylation level is equal for both substrates as is tested for six different enzyme preparations This latter finding is apparently in contrast with findings of Jackson and Saccomani (12) who found that the $EP_{(Pi)}$ level was twice the $EP_{(ATP)}$ level, although it is not clear whether the levels mentioned in their study can be compared

The dephosphorylation rate of the phosphoenzymes formed by ATP and P_i is for both substrates increased by K^+ Although each observation alone does not give perfect evidence it is obvious from the

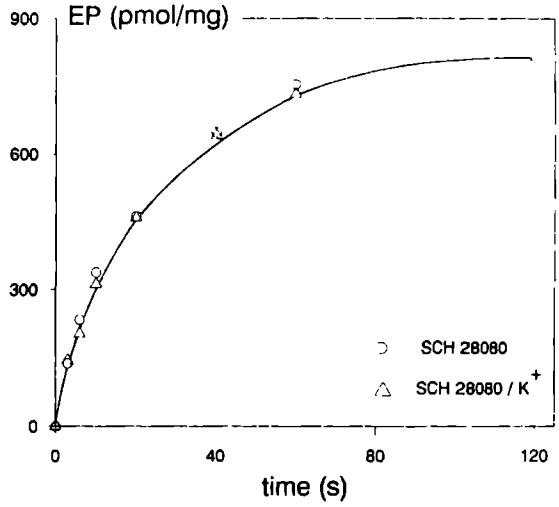

Figure 8. ***Time course of P_i phosphorylation with SCH 28080 and K^+***
H,K-ATPase (150 µg/ml) was phosphorylated with 60 µM $^{32}P_i$ in the presence of 1 mM Mg^{2+}, 50 mM imidazole/HCl (pH 6 0), 0 1 mM SCH 28080 with (Δ) or without 10 mM K^+ (O) The reaction was stopped after the indicated time intervals and the level of the phosphorylated intermediate (EP) was determined

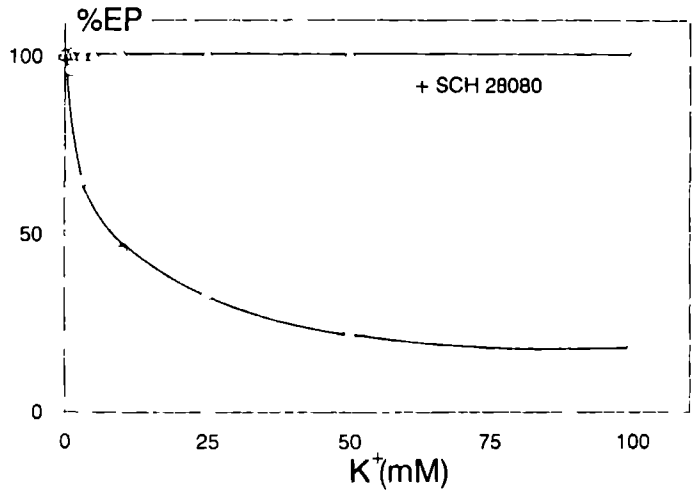

Figure 9. ***Abolishment of the K^+ effect on the steady state phosphorylation level by SCH 28080***
H K-ATPase (150 µg/ml) was preincubated with 1 mM Mg^{2+}, 50 mM imidazole/HCl (pH 6 0) and varying K^+ concentrations for 30 min Thereafter SCH 28080 (Δ) or vehicle (O) was added and the preincubation was continued for a further 30 min Subsequently $^{32}P_i$ was added to a final concentration of 60 µM The reaction was stopped after 60 s and the level of the phosphorylated intermediate (EP) was determined

whole of the arguments that $EP_{(P)}$ and $EP_{(ATP)}$ are identical

K^+ effects on the P_i phosphorylation and dephosphorylation

In Figure 10 a reaction scheme is given which fits the results The K^+ stimulated dephosphorylation reaction is indicated as reaction 1

In this scheme the binding of K^+ to the high affinity K^+ binding site of the enzyme yielding E_2K^+ is represented by reaction -2 The subsequent P_i phosphorylation reaction is marked -1 This reaction has a $K_{0.5}$ for K^+ of 0 3 mM which is in the same range as the $K_{0.5}$ for K^+ for the dephosphorylation reaction (reaction 1) (cf Figure 3 and 4) The increase in the exchange of ^{18}O between ^{18}O labeled P_i and oxygen of the enzyme by K^+, as observed by Faller and Diaz (24), probably results from the higher rates of the phosphorylation and the dephosphorylation reactions caused by K^+ The rate increasing effect of K^+ must be due to rapid binding of K^+ since the steady state phosphorylation level was reached within 3 s (Figure 6)

Extrapolation of the reaction rate as a function of the K^+ concentration yielded a rate constant for the P_i phosphorylation at 0 mM K^+ of 0 06 s^{-1} The rate constant for the dephosphorylation reaction in the absence of K^+ was in the same range Therefore one can conclude that there is a basal K^+ independent phosphorylation and dephosphorylation reaction The basal P_i phosphorylation and dephosphorylation reactions are indicated 3 and 3, respectively The rate constants of these reactions must be considerably smaller than these of the reactions in the presence of K^+ (-1 and 1), which approach values of 0 3 s^{-1} at infinite K^+ concentrations

At higher K^+ concentrations the steady state phosphorylation level is reduced ($K_{0.5}$ is 2 4 mM) in a noncompetitive fashion This effect can tentatively be explained by binding of K^+ to a low affinity binding site which, in the occupied state ($K^+E_2K^+$ in Figure 10), withdraws the enzyme from phosphorylation with P_i This reaction (reaction -4) is extremely slow since the steady state phosphorylation level is only reached after 2 min

The initial rapid increase of the phosphorylation level shown in Figure 7 is due to binding of K^+ to the high affinity K^+ binding site causing a larger portion of the enzyme being in the E_2K^+ form and thus an increase of the rate of reactions 1 and -1 Subsequently the decrease of the phosphorylation level caused by the binding of K^+ to the low affinity K^+ binding site (reaction -4) becomes apparent

Preincubation with high K^+ long enough to give K^+ the opportunity to bind to the low affinity K^+ binding site leads to an equilibrium between $K^+E_2K^+$ and E_2K^+ Therefore no transient is seen in the subsequent phosphorylation (Figure 7)

SCH 28080 effects on the P_i phosphorylation and dephosphorylation reactions

SCH 28080 inhibits the K^+ stimulated ATPase activity, H^+ secretion, para-nitrophenylphosphatase activity of H,K-ATPase by a competitive interaction with the K^+ site (20,25,26) It also inhibits the ATP phosphorylation reaction (20,25,26) In this study however no inhibition but an unexpected stimulatory effect of SCH 28080 was observed on the steady state phosphorylation level obtained with P_i, due to an increase in the affinity of the enzyme for P A similar effect was observed for ouabain and Na,K ATPase by Askari et al (27) These authors showed that ouabain stimulates the phosphorylation reaction of Na,K-ATPase with P It is postulated that the binding of ouabain to Na,K ATPase generates an E_2 form (28) SCH 28080 bound to the enzyme might in a similar way also generate an E_2 form of the enzyme

SCH 28080 abolishes the K^+ effects on the P_i phosphorylation rate constant, on the

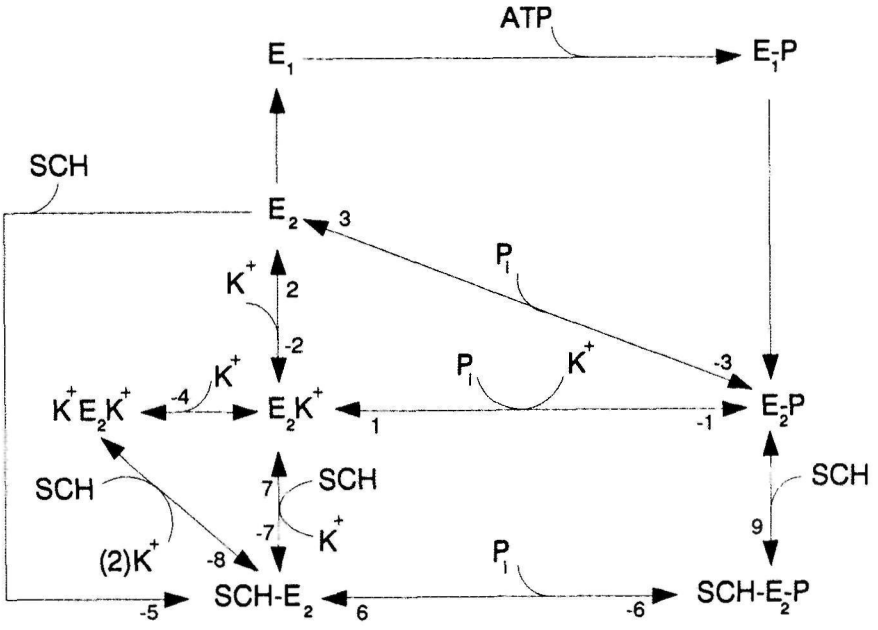

Figure 10. ***Putative reaction mechanism of the catalytic activities of H,K-ATPase***
The reactions depicted clockwise are indicated with a possitive number, the anti clockwise depicted reactions are indicated with a negative number. Details are described in the text.

steady state phosphorylation level and on the dephosphorylation reaction as can be expected from the fact that SCH 28080 binds to the K^+ binding site (21)

The P_i phosphorylation of the SCH 28080 bound enzyme is also represented in Figure 10 preincubation of the enzyme with SCH 28080 yields SCH-E_2 (reaction -5), phosphorylation of this compound with P_i gives SCH-E_2-P (reaction -6) The rate constant of reaction -6 is much lower than that of reaction -1 and equal to the rate constant of reaction -3

The abolishment by SCH 28080 of the rate increasing effect of K^+ on the phosphorylation reaction is also explained in Figure 10 Preincubation of H,K-ATPase with low K^+ concentrations results in the formation of E_2 K^+ (reaction -2). Addition of SCH 28080 replaces K^+ resulting in SCH-E_2 (reaction -7) Subsequent phosphorylation with P will also give SCH-E_2-P (reaction -6)

The lowering effect of K^+ on the steady state phosphorylation level is abolished by SCH 28080 as well This indicates that SCH 28080 influences both the high and the low affinity K^+ binding site By preincubating the enzyme with high K^+ concentrations $K^+E_2K^+$ is formed (reactions -2 and 4 successively) By addition of SCH 28080 subsequently to K^+, the latter ion is removed from its binding site resulting in the formation of SCH-E_2 (reaction -8), the phosphorylation with P_i proceeds as described by reaction -6 When the enzyme is preincubated with SCH 28080 in the absence of K^+ followed by addition of P_i reactions -5 and -6 take place successively Simultaneous addition of K^+ with P_i does not influence this order of reactions, as is clear from the absence of the rate stimulating effect of K^+

SCH 28080 also abolishes the effect of K^+ on the dephosphorylation rate This K^+ effect affects both the P_i and ATP phosphorylated enzyme. In the case of the ATP phosphorylated enzyme it should be noted that SCH 28080 should be added after the phosphorylation reaction but prior to the addition of K^+ This is due to the slow binding of SCH 28080 (21) in comparison to that of K^+ This explains why Wallmark et al (20) found no effect of SCH 28080 added simultaneously with K^+. If SCH 28080 is added prior to K^+ it will bind to the phosphorylated enzyme and give SCH-E_2-P (reaction 9) and will then be dephosphorylated in a K^+ independent way (reaction 6)

In the study towards the dephosphorylation reaction of the P_i phosphorylated enzyme SCH 28080 was present during the phosphorylation reaction and so SCH-E_2-P was formed The dephosphorylation reaction proceeds even in the presence of K^+ according to reaction 6.

Wallmark et al (20) suggested in contrast to Keeling et al (21) that SCH 28080 only binds to the E_2 form and not to the E_2-P form Our results indicate that the latter suggestion is incorrect P_i-phosphorylation can be carried out in the presence of SCH 28080 ATP-phosphorylation is inhibited by SCH 28080, but after phosphorylation with ATP, SCH 28080 binds to the phosphorylated intermediate as is shown by the fact that the intermediate becomes insensitive towards K^+. This is also the case when $EP_{(Pi)}$ is formed SCH 28080 therefore can bind both to phosphorylated and unphosphorylated forms of the enzyme

ACKNOWLEDGEMENTS

This investigation was financially supported by the Netherlands Organisation for Scientific Research (N W O), through the Netherlands Biophysics Foundation

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Chapter 10

General discussion and summary

The phosphorylation reaction and reconstitution of transport ATPases

In order to gain better insight in the mechanism of two closely related cation transport enzymes (Na^++K^+)-ATPase and (H^++K^+)-ATPase some aspects of the phosphorylation reaction have been studied. The (auto-) phosphorylation reaction of cation transporting enzymes plays a pivotal role in the reaction cycle of these membrane bound enzymes and can provide us a great deal of information about the working mechanism of the pump as a whole. Measurements of the steady-state phosphorylation level under different conditions mirrors the state of the enzyme. There are, however, some drawbacks linked to phosphorylation studies. Under most conditions the rate of the dephosphorylation reaction is measurable, but the rate of the formation of the phosphointermediate can not be determined without the aid of an advanced rapid mixing apparatus. Moreover, the steady-state phosphorylation level is a combination of several kinetic parameters and chemical equilibria which make exact interpretations difficult.

Some of the characteristics of membrane bound enzymes cannot be studied in the classical way, i.e. in isolated membrane fragments, since the compartimentalisation is lost in such a preparation. With the use of reconstitution procedures some of the features which are lost in the purified preparation can be restored.

Since the sidedness of ligands in the phosphorylation reaction of (Na^++K^+)-ATPase was one of the subjects of study, this enzyme was incorporated in a closed bilayer (liposome). In order to make current measurements of the pumped protons possible (H^++K^+)-ATPase was reconstituted onto a planar bilayer. In the latter reconstituted system it was possible to study the H^+ translocation step which occurs concomitantly with the phosphorylation reaction. Experiments with a substrate analogue were focussed on the nature of substrate specificity of the phosphorylation reaction and subsequent reactions in the pump cycle in which ATP is involved. The latter experiments were carried out with purified membrane fragments containing the transport ATPases. To determine whether the hydrolysis of the substrate analogue had anything to do with ion transport the latter feature was tested both with native gastric membrane vesicles containing (H^++K^+)-ATPase and with (Na^++K^+)-ATPase reconstituted in proteoliposomes. The phosphorylation reaction of (H^++K^+)-ATPase by inorganic phosphate which plays no direct role in cation transport under physiological conditions has been studied with an unsided preparation of gastric (H^++K^+)-ATPase.

Reconstution of (Na^++K^+)-ATPase

Since purified (Na^++K^+)-ATPase is obtained as membrane sheets in which the protein is surrounded by lipids from the bilayer in a disc like structure, the most important feature of the enzyme, namely its transport capacity, has been lost. Incorporation of the purified enzyme in vesicles of known lipid composition (reconstitution) restores this characteristic. In contrast to the purified enzyme which is unsided the reconstituted enzyme spans the membrane and faces two compartments so that the sidedness of effects of ligands can be studied in this system. Reconstituted (Na^++K^+)-ATPase has been used in this thesis to study effects of cations and amine compounds on the phosphorylation reaction at either side of the membrane.

Since the reconstituted (Na^++K^+)-ATPase is shown to be capable of transmembraneous cation transport (Chapter 2) it is obvious that the enzyme must span the bilayer as is the case in the native situation. In chapter 3 the reconstituted system

has been used to study the lipid dependency of $(Na^+ + K^+)$-ATPase with respect to cholesterol and the polar headgroups of some phospholipids.

Lipid dependency of reconstituted $(Na^+ + K^+)$-ATPase

In the literature a long lasting dispute is going on on the lipid dependency of $(Na^+ + K^+)$-ATPase. There are arguments for and against the absolute requirement of cholesterol and negatively charged phospholipids for the activity of $(Na^+ + K^+)$-ATPase.

Reconstitution of the parially solubilized $(Na^+ + K^+)$-ATPase with the freeze-thaw-sonication procedure in vesicles containing cholesterol and phosphatidylcholine as the only phospholipid yielded active pumping $(Na^+ + K^+)$-ATPase (Chapter 2), indicating that negatively charged phospholipids are not absolutely required for activity of $(Na^+ + K^+)$-ATPase.

Replacement of phosphatidylcholine by phosphatidylethanolamine did not alter the molar activity of the ATPase neither in the absence nor in the presence of K^+. The presence of negatively charged phospholipids (phosphatidylserine, phosphatidic acid and phosphatidylinositol) increased the molar activtiy of $(Na^+ + K^+)$-ATPase markedly. The results indicate that the presence of negatively charged phospholipids increases the activity of $(Na^+ + K^+)$-ATPase, although they are not an absolute requisite.

A controversy also exists on the role of cholesterol as an activating lipid compound of $(Na^+ + K^+)$-ATPase. Some authors claim it to be absolutely necessary for enzyme activity, whereas other authors state that it is of no importance for it. In the reconstituted system the role of cholesterol appeared to be dependent on additives present during the reconstitution procedure and the manipulations of the reconstitution procedure per se. When care was taken that during the reconstitution method inactivation of the enzyme was as low as possible, cholesterol did not influence the activity of the reconstituted $(Na^+ + K^+)$-ATPase. Cholesterol appeared to protect the enzyme against inactivation mainly during the sonication step in the reconstitution procedure.

The experiments concerning lipid dependency of $(Na^+ + K^+)$-ATPase in the older literature were carried out according to mainly two strategies: i. delipidation of the enzyme with detergents or organic solvents or ii. enzymatic modification of certain lipids. The first menthod has the disadvantage of (partially) denaturation (inactivation) of the enzyme and the observed effects may be rather restoration of the activity than activation of the enzyme. The second method has the disadvantages of unknown side effects of the enzymes and the possibility that some of the lipids are not readily attacked by the enzyme. The lysophosphatidyl compounds formed by the action of phospolipase A_1 or A_2 may influence the enzyme activity. Inactivating effects on the enzyme by the other breakdown products of the phospholipids, the unesterified fatty acids, have been demonstrated (Huang, W.H., Kakar, S.S. and Askari, A. (1986) Biochem. Int. 12, 521-528).

The mechanism of activation by the negatively charged phospholipids is hitherto unknown, but it may be possible that electrostatic binding of the headgroups to negatively charged amine groups helps to reactivate the enzyme by restoration of the tertiary structure of the enzyme which is lost in the delipidation procedure.

Sidedness of ligands involved in the phosphorylation reaction

It has been postulated several years ago that a cytoplasmic Na^+ site and an extracellular K^+ site are important in the phosphorylation and dephosphorylation reaction of the enzyme, respectively. Binding of cytoplasmic Na^+ is supposed to induce a conformational state of the enzyme which has a high affinity for ATP and can be phosphorylated in the presence of Mg^{2+}. K^+ accelerates the rate of the dephosphorylation reaction and therefore the overall activity of the enzyme. Whether there are more binding sites of these three physiologically important cations and what role they play in the reaction mechanism of $(Na^+ + K^+)$-ATPase has not been studied in much detail up till now, although evidence for multiple binding sites for physiological important cations

have been suggested (Askari, A. (1988) Prog. Clin. Biol. Res. 268A, 149-165). Reconstituted (Na^++K^+)-ATPase provides a suitable system for such a study since the ionic composition at both sides of the membrane can be controled and the influence on the phosphorylation reaction can be determined.

Apart from the already postulated sites for cytoplasmic Na^+ and extracellular K^+, several binding sites for these two monovalent cations have been observed (Chapter 4). Only one cytoplasmic Na^+ binding site could be observed with reconstituted (Na^++K^+)-ATPase used in this study, although additional binding sites for this cation have been suggested (Glynn, I.M. (1988) Prog. Clin. Biol. Res. 268A, 435-460). The failure for identification of additional Na^+binding sites in this study may be due to the resolution of the extracellular effects, resulting from different proteoliposome batches. An alternative explanation might be that the suggested binding sites are not involved in the phosphorylation reaction. An extracellular Na^+ binding site appeared to be important in the phosphorylation reaction. When this site was not occupied, only a very low phosphorylation level was obtained under optimal cytoplasmic conditions. A probable role of this site is to induce a conformational state of the enzyme which is able to bind ATP and to a subsequent phosphorylation reaction. A similar observation has already been reported (Karlish, S.J.D. and Stein, W.D. (1985) J. Physiol. 359, 119-149; Cornelius, F. and Skou, J.C. 1988 Biochim. Biophys. Acta 944, 223-232). According to the concept in which the transition to the E_1 conformation is induced by cytoplasmic as well as by extracellular Na^+ in a two-step mechanism, the role of cytoplasmic Na^+ is to trigger the phosphorylation reaction by ATP by changing the conformation induced by extracellular Na^+ towards the E_1 conformation. With the unsided preparation the role of Na^+ in the phosphorylation reaction was already known and supposed to be due to the cytoplasmic binding site. However, the extracellular binding site has not been acknowleged in experiments with fragmented enzyme. With a reconstituted preparation of (Na^++K^+)-ATPase with low extracellular Na^+ a low hydrolytic activity was reported which was explained by the low turnover rate of the phosphorylated enzyme (Cornelius, F. and Skou, J.C. (1988) Biochim. Biophys. Acta 944, 223-232). These authors did not actually measure the phosphorylation level and it can be argued that the low activity was due to a low phosphorylation level.

Binding of extracellular K^+ reduced the steady-state phosphorylation level, but this reduction was less than expected on basis of studies with unsided preparations. This discrepancy can tentatively be explained by a hypothesis of Klodos and Nørby (Nørby, J.G., Klodos, I. and Christiansen, N.O. (1983) J. Gen. Physiol. 82, 725-759; Klodos, I. (1988) Prog. Clin. Biol. Res. 268A, 315-320) that K^+ blocks the conformational state of E_1P to E_2P. When this effect is due to extracellular K^+ the effect on the steady-state phosphorylation level due to the increase of the dephosphorylation rate could be obscured by an accumulation of the E_1P conformer. An alternative explanation for the relative insensitivity for extracellular K^+ is suggested by the retardation of the E_1P to E_2P conformation caused by a positive potential of the proteoliposomes (Rephaeli, A., Richards, D.E. and Karlish, S.J.D. (1986) J. Biol. Chem. 261, 12437-12440). This retardation may result in accumulation of the K^+ insensitive E_1P intermediate.

Apart from an extracellular high-affinity K^+ binding site the presence of a K^+ binding site with low affinity at the extracellular site was obtained from the phosphorylation experiments. K^+ bound to this site reduced the phosphorylation level probably by fixing the enzyme in an occluded form, preventing the conformational change to the E_1 form which is necessary for the high affinity ATP binding and subsequent phosphorylation. Occupation of this binding site by K^+ at low ATP concentrations may form a dead-end complex with two K^+ sites occupied. A high affinity K^+ site at the cytoplasmic side also reduces the phosphorylation level probably by induction of a shift in the phosphointermediates in the direction of dephosphorylation sensitive E_2P form, as has been proposed (Yoda, S. and Yoda, A. (1988) J. Biol. Chem. 263, 10320-10325). The reduction of

the phosphorylation level strongly depended on the conditions during the phosphorylation reaction. This explains why some authors did not observe the effect. The observation that erythrocyte (Na^++K^+)-ATPase, treated with anti-L antibodies, showed a decrease in activity in the presence of cytoplasmic K^+ (Dunham, P.B. (1988) Prog. Clin. Biol. Res. 268A, 493-500) is in line with the above described effects of cytoplasmic K^+. Occupation of low affinity cytoplasmic K^+ binding sites also leads to formation of an occluded E_2K conformation simular to that formed by extracellular K^+. The latter observations support an hypothesis of Glynn (Glynn, I.M. (1988) Prog. Clin. Biol. Res. 268A, 435-460) in which it is stated that K^+ can become occluded also via cytoplasmic sites in the presence of very low ATP concentrations.

The third important compound involved in the phosphorylation reaction is the divalent cation Mg^{2+}. It plays a role in the binding of the γ-phosphate group of ATP to the enzyme and must act therefore at the cytoplasmic side. No effects of extracellular Mg^{2+} have been observed with reconstituted (Na^++K^+)-ATPase, indicating that Mg^{2+} is only involved in the phosphorylation reaction at the cytoplasmic side. The observed cytoplasmic effects correlated well with those of experiments with unsided enzyme preparations.

Together with the physiologically important cations the role of amine compounds in the phosphorylation reaction has been studied with reconstituted (Na^++K^+)-ATPase (Chapter 5). Several effects, stimulatory as well as inhibitory, with complex kinetics have been reported in the literature from studies with unsided enzyme preparations (Schuurmans Stekhoven, F.M.A.H., 't Lam, G.K., Zou, Y.S. and De Pont, J.J.H.H.M. (1988) Biochim. Biophys. Acta 937, 161-176), but many uncertainties about the mechanism still exist. Moreover, the stimulatory and inhibitory effects obscured each other because they were observed with the same amine at different concentrations. By splitting the effects of these compounds geometrically, more insight into the mechanism was obtained.

All amine compounds tested except histidine increased the phosphorylation level from the extracellular side in a similar way as Na^+. From this observation it is concluded that the extracellular binding site for Na^+ can be occupied by other compounds with the same effect and this site is therefore not very specific. The role of amines, however, is not necessarily the same as that of Na^+. It was demonstrated that amines can occupy the extracellular K^+ (occlusion) site, without being occluded. An amine in such position might withdraw K^+ from entering its occlusion site (Forbush, B. III (1988) Prog. Clin. Biol. Res. 268A, 229-248. Both inhibitory and stimulatory effects were observed at the cytoplasmic side of the enzyme. The cytoplasmic effects had relatively low affinity compared to the extracellular effects with exception of the one for ethylenediamine.

From the results with reconstituted (Na^++K^+)-ATPase it was concluded that the stimulatory effects observed with the unsided enzyme preparation are due to extracellular effects leading to a conformation of the enzyme suitable for phosphorylation. The effect on the extracellular side is not sufficient for the phosphorylation reaction, but also needs a cytoplasmic trigger. This cytoplasmic trigger is normally the binding of Na^+ but can also be exerted by some amine compounds which are known to be stimulatory in the unsided preparation. The inhibitory amine compounds do not exert this Na^+ like effect and their inhibitory behaviour is probably due to competition with Na^+ for the cytoplasmic Na^+ site, without stimulation of the phosphorylation reaction, since they oppose the stimulatory effect of cytoplasmic Na^+. The complex kinetics and the mixture of inhibitory and stimulatory effects observed with unsided preparations of (Na^++K^+)-ATPase are partially simplified when the effects are spatially separated, although several (inhibitory) effects of amines in unsided preparations are still difficult to explain. Results from experiments with an unsided preparation of (Na^++K^+)-ATPase (Fukushima, Y. (1988) Prog. Clin. Biol. Res. 268A, 301-306) showed an additional effect of amines on this enzyme: in the presence of dithiothreitol and SDS some amines inactivated the enzyme. It was suggested that the

inactivation was due to binding of the amine compounds to hydrophobic domains of the enzyme. Since the hydrophobic domains of the enzyme are suposed to be shielded by the lipids surrounding it in the proteoliposome this effect will not become apparent in reconstituted $(Na^+ + K^+)$-ATPase.

Differences in substrate specificity for the phosphorylation reaction and the total reaction cycle of transport ATPases

Both $(Na^+ + K^+)$-ATPase and $(H^+ + K^+)$-ATPase show a biphasic behaviour in the hydrolysis reaction with respect to the substrate. For $(Na^+ + K^+)$-ATPase a low affinity binding site has been demonstrated. The function of this site is to accelerate the deocclusion of K^+ from the enzyme and to induce a conformational state which is suitable for phosphorylation.

For $(H^+ + K^+)$-ATPase ample evidence for a second substrate binding site has been reported (Faller, L.D. (1989) Biochemistry 28, 6771-6778) but a similar function as with $(Na^+ + K^+)$-ATPase of such a low affinity binding site has not been demonstrated up till now. From the biphasic behaviour, however, it may be concluded that a regulatory substrate binding site must exist for $(H^+ + K^+)$-ATPase as well.

With *lin*-benzo-ATP, an ATP analogue with the adenosine moiety spatial extended by the formal insertion of a benzene ring, the substrate specificity of the high and low affinity binding sites of $(Na^+ + K^+)$-ATPase and $(H^+ + K^+)$-ATPase has been studied.

The high affinity binding site for ATP of $(Na^+ + K^+)$-ATPase reacted quite similar to both, the natural and synthetic substrate. Differences were found in the affinity for the substrate and the phosphorylation level obtained with this substrate. With the synthetic substrate the phosphorylation level was reduced somewhat compared to that with ATP. With respect to the low affinity binding site, however, a great difference existed between *lin*-benzo-ATP and ATP. The synthetic substrate appeared to be incapable to deocclude K^+ from the enzyme and to induce the E_1 conformation. The rate of hydrolysis with *lin*-benzo-ATP was therefore very low compared to that with ATP.

A similar behaviour had already been observed with other analogues of ATP which are able to phosphorylate the enzyme, but unable to drive the hydrolytic reaction with a rate comparable to that of ATP (Fu, Y.F., Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., De Pont, J.J.H.H.M. and Bonting, S.L. (1985) Biochim. Biophys. Acta 817, 7-16). Part of the failure to induce the conformational change towards E_1 can be explained by the concept of Boldyrev et al. (Boldyrev, A.A., Lopina, O.D. and Svinukhova, I.A. (1988) Prog. Clin. Biol. Res. 268A, 279-286). These authors state that the capability to induce the conformational change depends on the proton accepting capacitiy of the substrate. Since *lin*-benzo-ATP has a mediocre pK_a value in the Boldyrev series, but is hydrolyzed with a rate far below the lowest one of this series, there must be another factor which determines the effectiveness with which the compound may induce the conformational change. Since the extended adenosine ring is the only other difference of this synthetic substrate with ATP the geometric properties of the substrate are considered to play also an important role at the low affinity substrate binding site. The spatial requirements of the high affinity binding site therefore appeared to be less strict than that of the low affinity binding site.

Furthermore, *lin*-benzo-ATP was not able to catalyze electrogenic cation transport by reconstituted $(Na^+ + K^+)$-ATPase as tested with the fluorescent membrane potential probe oxonol VI. This indicated that the hydrolysis of this substrate could not lead to electrogenic Na^+ and K^+ transport or to electrogenic Na^+ transport. This is in accordance with the observation that in the electrogenic Na^+ transport the role of K^+ is taken over by Na^+ (Cornelius, F. and Skou, J.C. (1988) Biochim. Biophys. Acta 943, 223-232). From the transport experiment it cannot be excluded that *lin*-benzo-ATP can catalyze the electroneutral Na^+-Na^+ exchange. It may however be concluded that the low affinity substrate binding site plays a crucial role in the physiological function of $(Na^+ + K^+)$-ATPase.

Similar results as with $(Na^+ + K^+)$-ATPase were obtained with Ca^{2+}-ATPase from sarcoplasmic reticulum. In contrast to $(Na^+ + K^+)$-ATPase, however, with Ca^{2+}-ATPase cation transport was observed which could be correlated with the hydrolysis of *lin*-benzo-ATP.

The substrate specificity for the high affinity substrate binding site of $(H^+ + K^+)$-ATPase is more stringent than that of $(Na^+ + K^+)$-ATPase. Although a phosphorylated intermediate is formed with *lin*-benzo-ATP, the rate of this formation is almost three orders of magnitude smaller than that for ATP. Once formed the phosphorylated intermediate is identical to that formed with ATP. An appreciable rate of hydrolysis of the synthetic substrate was observed. This rate cannot be explained by the phosphorylation of $(H^+ + K^+)$-ATPase by *lin*-benzo-ATP and subsequent dephosphorylation of the phosphointermediate, since the phosphorylation rate is too slow.

That the hydrolysis of *lin*-benzo-ATP is not involved in the reaction cycle of $(H^+ + K^+)$-ATPase was confirmed by the observation that this substrate was not able to catalyze H^+ and K^+ transport in gastric vesicles.

This leads to the conclusion that there must be a hydrolytic activity in the enzyme preparation which is not part of the H^+/K^+ transport cycle. This hydrolytic activity must be due to an impurity in the enzyme preparation, or (less probable) to a phosphatase activity of the enzyme which is not involved in the reaction steps of the transport cycle. Furthermore it can be concluded that the substrate specificity of the three transport ATPases is much different with respect to both high and low affinity binding sites, with increasing sensitivity in the series Ca^{2+}-ATPase< $(Na^+ + K^+)$-ATPase< $(H^+ + K^+)$-ATPase.

Proton translocation during the phosphorylation step of $(H^+ + K^+)$-ATPase

Proton transport can be measured with a sensitive pH electrode or with pH sensitive fluorescent probes in native or synthetic vesicles containing $(H^+ + K^+)$-ATPase. From these experiments a 1:1 ratio for H^+:K^+ has been obtained by several groups (Skrabanja, A.T.P., Van der Hijden, H.T.W.M. and De Pont, J.J.H.H.M. (1987) Biochim. Biophys. Acta 903, 434-440). It was therefore concluded that the overall reaction cycle of $(H^+ + K^+)$-ATPase is electroneutral. Reconstitution of membrane bound transport enzymes to a planar membrane provides a system in which pump currents of the enzymes can be measured via the capacitive coupling between the membrane sheets and the planar bilayer (Fendler, K., Grell, E., Haub, M. and Bamberg, E. (1985) EMBO J. 4, 3079-3085). Since the $(H^+ + K^+)$-ATPase pumping cycle is electroneutral, no pump currents could be observed under physiological conditions (i.e. in the presence of K^+) with $(H^+ + K^+)$-ATPase reconstituted in this system. By kinetic separation of the phosphorylation step (with concomitant H^+ translocation) from the subsequent reaction steps (including the K^+ counter transport) a transient pump current was observed upon a concentration jump of ATP in the cuvette. ATP was released from caged-ATP by illumination of the non-hydrolysable substrate by means of an UV-flash. It was demonstrated in several experiments (Chapter 8) that the observed pump current originated from the proton translocation which occurs concomitantly with the phosphorylation step. From the experiments it could be concluded that the reaction cycle of $(H^+ + K^+)$-ATPase fits in a consecutive reaction scheme as proposed for $(Na^+ + K^+)$-ATPase by Albers and Post.

Because of the electroneutrallity of the overall reaction cycle of $(H^+ + K^+)$-ATPase the K^+ counter transport has to be electrogenic too. This feature of the reaction cycle is in contrast with $(Na^+ + K^+)$-ATPase, where only the Na^+ transport is voltage dependent and therefore probably electrogenic (Goldschlegger, R., Karlish, S.J.D., Rephaeli, A. and Stein, W.D. (1987) J. Physiol. 387, 331-355; De Weer, P. Gadsby, D.C. and Rakowski, R.F. (1988) Prog. Clin. Biol. Res. 268A, 421-434; Borlinghaus, R.T., Apell, H.-J. and Läger, P. (1988) Prog. Clin. Biol. Res. 268A, 477-484) and the K^+ translocation translocation electroneutral (Bahinski, A., Nakao, M. and Gadsby, C., (1988) Proc. Natl. Acad. Sci. USA 85, 3412-3416). For $(Na^+ + K^+)$-ATPase it was

postulated that two Na^+ were carried on a negatively charged (carboxyl-) group, whereas a third one is bound to a neutral site during the translocation step. This third ion causes the net charge transport, since K^+ is also bound to negatively charged groups during transmembrane transport (Karlish, S.J.D., Goldschlegger, R., Shahak, Y. and Rephaeli, A. (1988) Prog. Clin. Biol. Res 268A, 519-524). Similarly for $(H^+ + K^+)$-ATPase it must be concluded that both H^+ and K^+ are bound to neutral sites during ion transport.

The planar bilayer setup was also well suited for the study of the of the mechanism of a specific inhibitor of $(H^+ + K^+)$-ATPase, omeprazole (Lindberg, P., Nordberg, P., Alminger, T., Brändström, A. and Wallmark, B. (1986) J. Med. Chem. 29, 1327-1329). By addition of omeprazole to the protein-free compartment of the cuvette it was demonstrated that the hydrophobic (unprotonated) form of the inhibitor could penetrate the membrane and was activated by protonation in close proximity of the enzyme. By dissipating the protons from the interstitial phase, the activation of the inhibitor was not changed. It was concluded that the activation (protonation) of the inhibitor was not necessarily due to protonation in the medium, but could also be due to protonation by exposure to acidic groups of the enzyme.

Phosphorylation of $(H^+ + K^+)$-ATPase by inorganic phosphate

Like $(Na^+ + K^+)$-ATPase $(H^+ + K^+)$-ATPase can be phosphorylated by inorganic phosphate (P_i). This phosphorylation reaction is the reverse of the dephosphorylation reaction of the E_2P intermediate, formed by ATP (Faller, L.D. and Diaz, R.A. (1989) Biochemistry 28, 6908-6914). Various pieces of evidence are presented in Chapter 8 that the phosphorylated termediin ate is the same aspartyl phosphate as obtained by phosphorylation with ATP (Walderhaug, M.O., Post, R.L., Saccomani, G., Leonard, R.T. and Briskin, D.P. (1985) J. Biol. Chem. 260, 3852-3859). The phosphorylation reaction by P_i appeared to be very slow, but could be accelerated by low (micromolar) concentrations of K^+. These K^+ concentrations not only accelerate the phosphorylation reaction by P_i, but also the dephosphorylation reaction of the phosphointermediate formed by ATP as well as P_i. Higher K^+ concentrations in the millimolar range reduced the phosphorylation level of the phosphoenzyme phosphorylated by P_i, probably by forming a dead end complex by binding K^+ to low affinity binding sites. Similar results were obtained in phosphorylation experiments with reconstituted $(Na^+ + K^+)$-ATPase (Chapter 4).

The maximal phosphorylation level of 1.5 nmol phosphate bound per mg protein obtained by P_i as substrate is equal to that obtained with ATP. This observation is apparently in conflict with results of Jackson and Saccomani (Jackson, R.J. and Saccomani, G. (1984) Biophys. J. 45, 83a), who reported a phosphorylation level of 2.5-2.7 nmoles phosphate per mg protein. It has been suggested that the level obtained with P_i is about two times higher as the highest steady-state phosphorylation level obtained with ATP as the substrate. This level would be comparable with that of vanadate, AMP-PNP, TNP-ATP, FITC, eosin and omeprazole binding which is about 3 nmoles per mg protein. Since Jackson and Saccomani did not mention the conditions under which the phosphorylation reaction was carried out and since they did not provide details of the protein assay, some care must be taken with the meaning of value of this level of 2.7 nmoles per mg. Moreover, the inhibition by vanadate and the binding of vanadate and TNP-ATP were reported to be biphasic (Faller, L.D., Rabon, E. and Sachs, G. (1983) Biochemistry 22, 4676-4685; Faller, L.D. (1989) Biochemistry 28, 6771-6778). Binding sites with both, high and low affinity with a binding capacitiy of 1.5 nmoles per mg were derived indicating that two binding sites were involved. It is possible that the high phosphorylation levels obtained by Jackson and Saccomani resulted from binding of P_i to the low affinity vanadate site, since the high levels were obtained with extreme high levels of P_i (5 mM) (Faller, L.D. and Elgavish, G.A. (1984) Biochemistry 23, 6584-6590). The binding capacity of the phosphointermediate obtained with ATP and P_i may well corellate with one of these two substrate bindings sites. From

the molecular weight of the holoenzyme it must then be concluded that maximally one out of three or four subunits of $(H^+ + K^+)$-ATPase is phosphorylated and therefore has to be considered a catalytic one. The holoenzyme is in this view probably a tri- or tetramer.

All effects of K^+ on the P_i phosphorylation were counteracted by the specific inhibitor of $(H^+ + K^+)$-ATPase, SCH 28080 (Beil, W. Staar, U. and Sewing, K.-F. (1987) Eur. J. Pharmacol. 139, 349-352). Beside this antagonistic effect with respect to K^+ the drug also increased the affinity of the enzyme for P_i by a factor of three. The inhibitor also increased the level of the phosphorylated intermediate slightly (12%). Perhaps a low affinity site equivalent to the low affinity vanadate binding site is (partially) exposed under the influence of SCH 28080. From these experiments it was concluded that in contrast to observations in a recent report (Wallmark, B., Briving, C., Fryklund, J., Munson, K., Jackson, R., Mendlein, J., Rabon, E. and Sachs, G. (1987) J. Biol. Chem. 262, 2077-2084) SCH 28080 not only binds to the dephosphoenzyme, but also to the phosphorylated E_2 conformation of the enzyme.

Most important findings described in this thesis

$(Na^+ + K^+)$-ATPase contains besides a cytoplasmic Na^+ site a Na^+ site at the extracellular side which plays an important role in the phosphorylation reaction of the enzyme.

The role of the extracellular Na^+ is not very specific and can be overtaken by several positively charged amine compounds.

$(Na^+ + K^+)$-ATPase has not only a high affinity K^+ binding site at the extracellular side of the membrane, but also low affinity extracellular and low and high affinity cytoplasmic K^+ binding sites which play a role in the phosphorylation reaction of the enzyme.

Negatively charged phospholipids increase the molar activity of $(Na^+ + K^+)$-ATPase but are not an absolutele requirement.

Cholesterol is not required for the activity of $(Na^+ + K^+)$-ATPase, but plays a protective role against inactivation of the enzyme during the reconstitution procedure.

The high affinity substrate binding site of transport ATPases has a lower substrate specificity than the low affinity binding site.

The K^+ insensitive basal Mg^{2+}-ATPase activity of $(H^+ + K^+)$-ATPase is not involved in the transport cycle of the enzyme.

The reaction cycle of $(H^+ + K^+)$-ATPase fits in the consecutive Albers-Post scheme, originally postulated for $(Na^+ + K^+)$-ATPase.

The K^+ translocating step of $(H^+ + K^+)$-ATPase is in contrast to $(Na^+ + K^+)$-ATPase electrogenic.

The slow rate of the phoshorylation reaction of $(H^+ + K^+)$-ATPase by inorganic phosphate can be increased by low concentrations of K^+, without decreasing the phosphorylation level.

The effects of high and low K^+ concentrations in the phosphorylation reaction of $(H^+ + K^+)$-ATPase with inorganic phosphate can totally antagonized by the specific inhibitor of $(H^+ + K^+)$-ATPase SCH 28080.

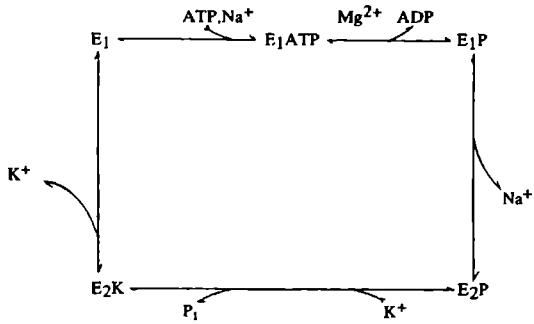
SAMENVATTING

De fosforyleringsreactie van transport ATPases. Een onderzoek met gezuiverde en gereconstitueerde enzymen.

In dit proefschrift wordt het onderzoek naar enkele aspekten van het werkingsmechanisme van twee membraan gebonden cation transporterende enzymen (Na^++K^+)-ATPase en (H^++K^+)-ATPase beschreven, waarbij de fosforyleringsreaktie centraal staat. (Na^++K^+)-ATPase komt in de plasmamembraan van alle zoogdiercellen voor en zorgt voor aktief transport van K^+ de cel in en Na^+ de cel uit. Dit leidt tot handhaving van gradienten en concentraties van ionen, die nodig zijn voor vele biochemische en fysiologische processen in de cel. (H^++K^+)-ATPase komt hoofdzakelijk voor in de parietale cel in de maag en zorgt door een aktieve uitwisseling van H^+ met K^+ voor een lage zuurgraad in de maag, welke nodig is voor de werking van spijsverterings enzym pepsine.

De beide ATPases vertonen struktureel en mechanistisch sterke overeenkomsten. De belangrijkste katalytische subeenheid van beide ATPasen bestaat uit een 100 kDa polypeptide, met een sterke homologie in primaire structuur.

De reactiecyclus van beide ATPases kan worden herleid tot onderstaand simpel schema.



Het eenmaal doorlopen van deze cyclus resulteert in transport van kationen over de membraan en de hydrolyse van een molekuul ATP. De chemische energie die vrijkomt bij de splitsing van ATP wordt gebruikt voor het transport van kationen tegen electrochemische gradienten in. ATP wordt met hoge affiniteit gebonden aan de E_1 vorm van het enzym dat ook met hoge affiniteit Na^+ (of H^+ in het geval van (H^++K^+)-ATPase) bindt aan de cytosolische zijde van de membraan. Onder invloed van Mg^{2+} kan het enzym gefosforyleerd worden (E_1P). Na conformatieveranderingen van het gefosforyleerde enzym, die gepaard gaan met Na^+ translocatie over de membraan, neemt de affiniteit voor Na^+ (H^+) af waardoor dit cation wordt losgelaten aan de extracellulaire zijde (E_2P). Hier kan K^+ vervolgens met hoge affiniteit binden. Deze binding versnelt de defosforyleringsreactie (E_2P--->E_2 + P_i). De E_2 vorm met K^+ ondergaat een conformatie verandering waarbij K^+ naar de cytoplasmatische zijde wordt getransporteerd. De E_1 conformatie heeft een lage affiniteit voor K^+ waardoor dit kation loslaat van het enzym en Na^+ (H^+) weer kan binden zodat de cyclus rond is.

Gedurende vele jaren zijn strukturele en mechanistische eigenschappen van transportenzymen bestudeerd aan gezuiverde enzymen. Met verschillende zuiveringsmethoden worden de enzymen verkregen omringd door flarden lipidedubbellaag afkomstig van de plasmamembraan. Een belangrijke eigenschap van transportenzymen, namelijk het transport van kationen zelf, kan met deze preparaten niet meer worden bestudeerd daar geen onderscheid kan worden gemaakt tussen de twee zijden van het membraan. Om de enzymen hun oorspronkelijke eigenschappen weer (gedeeltelijk) terug te geven worden ze in

systemen gebracht waarin weer sprake is van twee te onderscheiden compartimenten (reconstitutie). Het (Na^++K^+)-ATPase werd gereconstitueerd in gesloten lipidedubbellagen (liposomen) zodat het mogelijk was de effecten van liganden op reacties in de cyclus te bestuderen. (H^++K^+)-ATPase werd gereconstitueerd aan een vlakke bilaag, die twee compartimenten scheidt. Door het meten van (pico-Ampère) stromen over de membraan kan de pompactiviteit van het enzym bestudeerd worden.

In hoofdstuk 2 worden verschillende procedures besproken om membraangebonden eiwitten te reconstitueren in lipidevesicles. De resultaten van deze methoden met (Na^++K^+)-ATPase, geisoleerd uit de konijnenier, werden vergeleken en de meest geschikte methode, de z.g. vris-dooi-sonicatie methode, werd geoptimaliseerd voor (Na^++K^+)-ATPase van de konijnenier. Met de geoptimaliseerde methode werd het (Na^++K^+)-ATPase tenslotte gereconstitueerd in proteoliposomen met relatief hoge fosforylerings-, hydrolyse- en transportcapaciteit.

Wanneer het enzym functioneel is ingebouwd in een lipidedubbellaag moet het transmembraneus gelokaliseerd zijn. De interactie van het eiwit met de lipiden is dan vergelijkbaar met de natuurlijke situatie. Daarom biedt dit systeem de mogelijkheid om de invloed van de lipidesamenstelling van de bilaag op de enzymaktiviteit te bepalen. Uit de experimenten zoals beschreven in hoofdstuk 3 bleek dat het enzym omgeven door uitsluitend neutrale fosfolipiden een (molaire) aktiviteit heeft die lager is dan die in aanwezigheid van de natieve lipiden, terwijl de aanwezigheid van negatief geladen fosfolipiden deze activiteit verhoogt tot ver boven die van het enzym omgeven door natieve lipiden. Bovendien bleek dat de aanwezigheid van cholesterol geen invloed te hebben op de enzymaktiviteit.

Om meer informatie te verkrijgen over bindingsplaatsen van de liganden en hun functie aan beide zijden van de membraan is met gereconstitueerd (Na^++K^+)-ATPase het effect bepaald op de fosforyleringsreaktie van de fysiologisch meest interessante liganden, te weten Na^+, K^+ en Mg^{2+} en van enkele amines. Uit de experimenten beschreven in hoofdstuk 4 bleek dat behalve de extracellulaire bindingsplaats met hoge affiniteit voor K^+ aan deze zijde ook nog een K^+-bindingsplaats met lage affiniteit voorkomt. Bovendien zijn K^+ bindingsplaatsen met hoge en lage affiniteit, welke betrokken zijn in de defosforyleringsreaktie van het (Na^++K^+)-ATPase gelokaliseerd aan de cytoplasmatische zijde. Bovendien zijn naast de cytoplasmatische Na^+-bindingsplaats ook extracellulaire Na^+ bindingsplaatsen aangetoond, die de fosforylering van het (Na^++K^+)-ATPase stimuleren. In hoofdstuk 5 wordt aangetoond dat deze functie van Na^+ niet bijzonder specifiek is daar positief geladen amines hetzelfde effect hebben als Na^+ aan de extracellulaire zijde. Enkele van deze amines, waarvan bekend is dat ze het fosforyleringsniveau van het niet-gereconstitueerde (Na^++K^+)-ATPase verhogen in afwezigheid van Na^+, kunnen de rol van cytoplasmatisch Na^+, zij het slechts in beperkte mate, overnemen. Daarentegen hadden enkele amines, die het Na^+-gestimuleerde fosforyleringsniveau van het niet-gereconstitueerde (Na^++K^+)-ATPase verlagen, ook een remmend effekt aan de cytosolische zijde van het gereconstitueerde (Na^++K^+)-ATPase. Door bestudering van de effekten van amines aan beide zijden van de membraan afzonderlijk, was het mogelijk waarnemingen uit experimenten met niet-gereconstitueerd (Na^++K^+)-ATPase te verklaren.

Voor (Na^++K^+)-ATPase en Ca^{2+}-ATPase is naast de substraatbindingsplaats met hoge affiniteit voor ATP, welke betrokken is bij de fosforylerings reaktie, ook een nucleotide-bindingsplaats met lage affiniteit aangetoond welke een rol speelt in de versnelling van de conformatieverandering van de E_2 naar de E_1 vorm. Met behulp van *lin*-benzo-ATP, een synthetisch ATP analoog, waarin in de adenosine ring is uitgebreid met een benzeenring, is aangetoond dat de hoog-affiene bindingsplaats een relatief lage substraatspecificiteit vertoont in vergelijking met de ATP-bindingsplaats met lage affiniteit (hoofdstuk 6).

In een soortgelijk onderzoek (hoofdstuk 7) bleek dat de de fosforyleringsreactie van het (H^++K^+)-ATPase met *lin*-benzo-ATP ruim 200 keer zo langzaam is dan met ATP. De fosforyleringssnelheid was dermate laag, dat de snelheid

van hydrolyse van het synthetische substraat niet veroorzaakt kon worden door fosforylering en opeenvolgende defosforylering van het $(H^+ + K^+)$-ATPase in de hierboven beschreven reactiecyclus. Hieruit kon gekonkludeerd worden dat de hydrolyse van het synthetische substraat, welke sterke overeenkomsten vertoont met de zogenaamde basale Mg^{2+}-ATPase activiteit, veroorzaakt moet worden door een verontreiniging in het enzym preparaat of door een fosfatase activiteit van het $(H^+ + K^+)$-ATPase zelf, welke niet betrokken is in de normale reaktiecyclus van het enzym.

Daar $(H^+ + K^+)$-ATPase onder fysiologische condities één K^+ uitwisselt tegen één H^+ is de pompcyclus van dit ATPase in tegenstelling tot $(Na^+ + K^+)$-ATPase electroneutraal. Electrogene eigenschappen kunnen derhalve onder natuurlijke omstandigheden niet worden gemeten. Met $(H^+ + K^+)$-ATPase, gereconstitueerd aan een vlakke lipidedubbellaag, konden in afwezigheid van K^+ echter transiente pomp stromen worden waargenomen (Hoofdstuk 8), die gerelateerd waren aan de fosforyleringsreactie. Daar de pompstromen afkomstig waren van de H^+-translocatie en deze niet werden waargenomen in aanwezigheid van K^+, werd bevestigd dat de pomp-cyclus van het $(H^+ + K^+)$-ATPase bestaat uit de translocatie van H^+ gevolgd door die van K^+, vergelijkbaar met de consecutieve reaktie-cyclus van $(Na^+ + K^+)$-ATPase. Bovendien moet gekonkludeerd worden, dat in tegenstelling tot het $(Na^+ + K^+)$-ATPase het transport van K^+ electrogeen is, daar deze stap de electrogene H^+ translocatie compenseert.

Net als $(Na^+ + K^+)$-ATPase ondergaat $(H^+ + K^+)$-ATPase behalve met ATP ook een autofosforyleringsreactie met anorganisch fosfaat (P_i). Deze reaktie welke kan worden beschouwd als het omgekeerde van de defosforyleringsreaktie van het fosfoenzym, was voor $(H^+ + K^+)$-ATPase in tegenstelling tot $(Na^+ + K^+)$-ATPase slechts summier beschreven in de literatuur. In Hoofdstuk 9 wordt de invloed van K^+ en een K^+-antagonist op de fosforyleringsreaktie van $(H^+ + K^+)$-ATPase met anorganisch fosfaat beschreven. De langzame fosforyleringsreaktie met anorganisch fosfaat werd, net zoals de defosforyleringsreaktie van het fosfointermediar, versneld door lage concentraties K^+, zonder dat het fosforyleringsniveau werd beinvloed. K^+ in hogere concentraties versnelde de defosforyleringsreaktie niet verder, maar verlaagde wel het fosforyleringsniveau. Alle effecten van K^+ op de fosforyleringsreaktie met P_i kunnen worden opgeheven met een specifieke remmer van $(H^+ + K^+)$-ATPase, SCH 28080. Dit duidt erop dat SCH 28080 beide typen K^+ bindingsplaatsen, met hoge en lage affiniteit, kan bezetten. Bovendien wordt het fosforylerings niveau van het $(H^+ + K^+)$-ATPase met P_i verhoogd door SCH 28080, doordat deze remmer de affiniteit voor P_i doet toenemen. Uit de experimenten mag derhalve gekonkludeerd worden, dat SCH 28080 in tegenstelling tot gegevens uit de literatuur bindt aan zowel de fosfo- als aan de defosfo E_2 vorm.

Tenslotte kan worden opgemerkt dat ondanks sterke overeenkomsten tussen het $(Na^+ + K^+)$-ATPase en $(H^+ + K^+)$-ATPase in dit proefschrift een aantal belangrijke verschillen zijn aangetoond tussen de twee transport ATPases zoals de substraatspecificiteit, de aard van de K^+-bindingsplaatsen en electrogene eigenschappen van de K^+-translocatie.

Harry T.W.M. van der Hijden werd geboren op 21 november 1957 te Schijndel. In 1976 behaalde hij het Atheneum-B diploma aan het Jacob Roelands Lyceum te Boxtel. Na een jaar Geneeskunde-studie aan de universiteit van Amsterdam en het behalen van het propedeutisch examen aldaar werd in september 1977 begonnen met de Biologie-studie aan de Katholieke Universiteit te Nijmegen. Na twee jaar werd deze studie gecombineerd met de studie Scheikunde, waarna in maart 1981 het kandidaatsexamen Scheikunde (S2) en in november van datzelfde jaar het kandidaatsexamen Biologie (B4) werden behaald. De doctoraalstudie omvatte als hoofdvakken Organische Chemie (Prof. Dr. B. Zwanenburg) en Biochemie (Prof. Dr. S.L. Bonting en Prof. Dr. J.J.H.H.M. De Pont) en als bijvak Chemische Microbiologie (Prof. Dr. G.D. Vogels). In juni 1985 werd het doctoraalexamen Scheikunde afgelegd gevolgd door het doctoraalexamen Biologie in november 1987.

Van oktober 1985 tot oktober 1989 was hij werkzaam aan het Laboratorium voor Biochemie van de Katholieke Universiteit te Nijmegen, in dienst van de Nederlandse Stichting voor Wetenschappelijk Onderzoek (N.W.O.) op een project van de Stichting voor Biofysica. Gedurende deze periode werd het in het proefschrift beschreven onderzoek verricht onder leiding van Prof. Dr. J.J.H.H.M. De Pont. Een deel van het onderzoek heeft hij uitgevoerd aan het Max-Planck Institut für Biophysik in Frankfurt, B.R.D. onder leiding van Prof. Dr. E. Bamberg. Gedurende zijn studie en promotieonderzoek was hij betrokken bij het onderwijs aan studenten Biologie, Geneeskunde en Gezondheidswetenschappen als assistent bij practica en heeft gedurende zijn promotieonderzoek drie doctoraalstudenten en twee HBO-stagaires begeleid.

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ISBN 90 9003 377 7